STUDIES OF POSSIBLE NEW APPROACHES TO THE TREATMENT OF HUMAN INSULIN-DEPENDENT DIABETES MELLITUS USING THE SPONTANEOUSLY DIABETIC, INSULIN-DEPENDENT AUTOIMMUNE BB/E RAT.

Christopher James Lynch

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DECLARATION

The experiments and composition of this thesis are, unless otherwise stated, my own work. No part of this work has been, or is being, submitted for any other degree, diploma or other qualification.

Christopher James Lynch
September 1994

DEDICATION

I dedicate this thesis with love and gratitude to my Mother and Father whose support, strength, encouragement and love enabled me to complete this PhD. I am totally indebted to you both.

ABSTRACT OF THESIS

This study investigated three possible new approaches to the treatment of human insulin-dependent diabetes mellitus (IDDM) using the insulin-dependent BB/E rat as an animal model of spontaneous diabetes.

Islets encapsulated in alginate-poly-L-lysine-alginate microcapsules and transplanted into animal models of IDDM may be protected from autoimmune rejection and destruction. Conflicting results have been reported upon testing the capacity of microencapsulated islets to secrete insulin in response to glucose challenge *in vitro*. The glucose-induced insulin secretory responses of (1) freshly isolated free islets and (2) free (i.e. non-encapsulated) and encapsulated islets cultured for different periods of time, in a multichannel perifusion system were therefore compared. Optimal insulin secretion of freshly isolated free islets was found to be significantly higher than that of free cultured and encapsulated cultured islets. A slight delay in response to glucose stimulus was observed in the encapsulated islet group. Insulin secretion by free cultured and encapsulated cultured islets was optimal after six and seven days culture respectively, and diminished thereafter. A recovery period appears critical for optimal function of encapsulated islet grafts.

In the second study, metabolic control and feeding patterns in spontaneously diabetic BB/E and streptozotocin-diabetic rats treated either conventionally with daily injections of insulin or using novel sustained release insulin implants were compared. Food consumption and plasma glucose concentration over a 24 hour period were measured and glycosylated haemoglobin determined in diabetic rats initially treated with daily injections of insulin and one month later following treatment with sustained release insulin implants, and compared with non-diabetic rats. Diabetic animals

maintained on conventional insulin treatment exhibited unstable plasma glucose profiles, and both random plasma glucose concentrations and glycated haemoglobin levels were inaccurate indices of overall metabolic control. In contrast, sustained release insulin implants achieved a relatively stable plasma glucose profile which was accurately reflected by these parameters. However, tissue concentrations of the principal metabolites of the polyol pathway, although decreased by both methods of insulin treatment, were not significantly different. Finally, non-diabetic rats consumed significantly more food during the twelve hour dark cycle than the light cycle, whereas diabetic rats ate continuously throughout the 24 hour period, and consumed significantly more than non-diabetic rats, irrespective of type and treatment of diabetes.

In the final study, the effect of a short course (14 days) of treatment with non-depleting and depleting monoclonal antibodies specific for T lymphocyte subsets on preventing rejection and autoimmune destruction of intraportal islet allografts was investigated in BB/E rats with established, insulin-dependent diabetes. Treatment with a depleting monoclonal antibody specific for helper/inducer ($CD4^+$) T lymphocytes either alone or in combination with a depleting antibody directed against cytotoxic/suppressor ($CD8^+$) T cells was most effective in prolonging graft function. Treated animals with early graft failure were compared with animals whose grafts functioned for a shorter or longer time in respect of changes in (1) T lymphocyte subsets in the circulation, peripheral and mesenteric lymph nodes, peritoneal exudate, spleen and thymus, (2) the proliferative ability of their peripheral blood lymphocytes (mitogen stimulation assay) and (3) hepatic, pancreatic and islet graft infiltration. Rats with a long-term functioning graft (64 - 91) days received a second donor-specific islet graft under the kidney capsule without further antibody treatment. Approximately 30 days later animals were killed and tissues, including the

islet-bearing kidney, were assessed as described above. FACS analysis of lymphocytes and lymphocyte subsets demonstrated that OX38 moAb exerted its immunosuppressive effect without significant depletion of $CD4^+$ cell numbers. Furthermore, there was no significant difference between the lymphocyte and lymphocyte subset numbers in the peripheral blood or lymphoid tissues of moAb-treated BB/E rats maintaining short- or long-term functioning islet grafts. Stimulation indices as a measure of the proliferative ability of PBL to Con A were decreased following moAb injection but no consistent finding to explain the prolonged survival of certain islet grafts over others was observed. However, analysis of lymphocytes and their subsets from peripheral blood and lymphoid tissues may not accurately reflect events within the islet allograft. All BB/E rats receiving a 2° islet graft under the kidney capsule remained normoglycaemic until death with the exception of a single animal which was borderline hyperglycaemic prior to 2° islet transplantation. Histologically, a chronic inflammatory cell infiltrate was observed within short-term functioning intraportal islet grafts and islets were weakly positive or negative for insulin, whereas a mild infiltrate was observed around islets that maintained long-term normoglycaemia which were strongly positive for insulin. In contrast to the long-term unresponsiveness to 1° islet grafts in moAb-treated BB/E rats, secondary islet grafts were not afforded the same protection.

LIST OF ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytoxicity
ALS	anti-lymphocyte serum
APA	alginate-poly-L-lysine-alginate
APC	antigen presenting cell
ARC	arcuate nucleus
AUC	area under curve
BSA	bovine serum albumin
CAMC	complement dependent antibody-mediated cytotoxicity
CIT	conventional insulin therapy
Con A	concanavalin A
CsA	cyclosporin A
DMN	dorsomedial nucleus
DP	diabetes-prone
DR	diabetes-resistant
EAE	experimental allergic encephalomyelitis
EAN	experimental allergic neuritis
ECACC	European Collection of Animal Cell Cultures
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
GAD	glutamic acid decarboxylase
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
I	ionomycin
IAA	insulin autoantibody
ICA	islet cell antibody
ICCA	islet cell cytoplasmic antibody
ICSA	islet cell surface antibody
IDDM	insulin-dependent diabetes mellitus
IEL	intraepithelium lymphocytes
IFN	interferon
Ig	immunoglobulin
IL-1	interleukin-1
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
IVGTT	intravenous glucose tolerance test
kD	kilodalton
LGL	large granular lymphocytes
MHC	major histocompatibility complex
MLN	mesenteric lymph nodes
MLR	mixed lymphocyte reaction
moAb	monoclonal antibody
NIR	near-infrared
NK	natural killer

NO	nitric oxide
NOD	non-obese diabetic
NPY	neuropeptide Y
PEC	peritoneal exudate cells
PBL	peripheral blood lymphocytes
PBS	phosphate-buffered saline
PLN	peripheral lymph nodes
PMA	phorbol myrisate acetate
PVN	paraventricular nucleus
RFLP	restriction fragment length polymorphism
RIA	radioimmunoassay
SRII	sustained release insulin implants
STZ	streptozotocin
TIC	total insulin content
TNF	tumour necrosis factor
UV	ultraviolet
WF	Wistar Furth
WH	Wistar Han
1°	primary
2°	secondary

PUBLICATIONS ARISING FROM WORK IN THIS THESIS

Lynch C J, Smith W, Lindsay R M, Baird J D. Achieving and assessing metabolic control in rats with spontaneous insulin-dependent autoimmune and streptozotocin-induced diabetes : comparison of metabolic control and feeding patterns in non-diabetic and diabetic rats treated conventionally with subcutaneous injections of insulin or sustained release insulin implants. To be submitted.

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LIST OF CONTENTS

CHAPTER1

INTRODUCTION

1.1. HUMAN AUTOIMMUNE INSULIN-DEPENDENT DIABETES MELLITUS

1.1.1. INTRODUCTION

The discovery of insulin, isolated from dog pancreas, by Banting and Best was initially announced to the Medical Faculty of the University of Toronto on the 11th November, 1921. Their findings were reported in the Journal of Laboratory and Clinical Medicine in February 1922 (1). Clinical trials using insulin in the treatment of human insulin-dependent diabetes mellitus (IDDM) began early in the same year, and the results published later in 1922 (2).

Subsequently pure crystalline insulin was commercially produced, and insulin therapy as a treatment for human diabetes was thought to have " cured " an otherwise fatal disease. Treatment with one or more injections of insulin per day, given into the subcutaneous tissue, controlled the symptoms of diabetes produced by acute elevation of blood glucose, including unassuageable thirst, profuse urination, lethargy and rapidly progressive body wasting, terminating in stupor, coma and death. Death from ketoacidosis was also greatly reduced.

It was almost 30 years before it was discovered that subjects thought to be " cured " of their diabetes by daily injections of insulin developed serious micro- and macroangiopathic complications of the disease (3). Neuropathy, nephropathy, retinopathy and vascular disease were particularly prevalent. After 20 years of diabetes, 80% of subjects had retinopathy and of these, approximately 50% were destined to die of kidney failure within another 10-15 years. This substantial

morbidity and mortality necessitated the need for an alternative " cure " for diabetes mellitus.

A " cure " for diabetes can be approached in two ways : primary prevention or a better method of treatment that allows the patient to lead a truly normal lifestyle and prevents the development of long term complications.

1.1.2. **AETIOLOGY OF HUMAN IDDM**

It is now generally recognised that genetic susceptibility plays a major role in the aetiology of IDDM. However, genetic studies have been hindered by several major obstacles, including variable age at onset of IDDM, the potential interaction of genetic and environmental factors, the lack of knowledge of the basic defect and the existence of genetic heterogeneity, i.e. separating IDDM from other forms of diabetes and the possibility of multiple forms of the disease.

1.1.2.1. HLA ANTIGENS

In man, genes of the major histocompatibility complex (MHC) called the HLA region are located on the short arm of chromosome 6, and encode for the three major classes of proteins : HLA-A, B and C encode class I molecules which act as cell surface recognition molecules; HLA-D contains genes for class II proteins which are involved in cooperation and interaction between cells of the immune system, and the region between HLA-D and B encodes class III proteins which are components of the complement system, including C2, factor B and C4a and C4b (4,5). These genes are located close to one another on chromosome 6 and thus are linked.

In man essentially all nucleated cells carry HLA-A, B and C class I proteins in varying amounts. Class I proteins consist of two polypeptides, the larger of which is encoded by the MHC and non-covalently associated with the polypeptide β_2-microglobulin, which is encoded outside the MHC. In contrast, the class II molecules encoded by HLA-D have a restricted distribution, and consist of two non-covalently associated peptides, the α- and β-chains, both of which are encoded by the MHC.

1.1.2.2. PATHOGENESIS

Three non-immunosuppressed diabetic twins receiving a segmental pancreas transplant from their non-diabetic co-twins, who were themselves unlikely to develop IDDM since they had been discordant for diabetes for more than 17 years, were temporarily cured of their diabetes. However, within a few weeks disease recurrence was observed as donor pancretic islets became infiltrated (insulitis) and graft functioning ceased (6). A fourth patient received a similar transplant under cover of the immunosuppressant azathioprine. No insulitis was observed and diabetes did not recur. Histolological examination of the failed pancreatic grafts demonstrated that, in contrast to a graft rejection response, only the islet insulin-producing β-cells were destroyed. This destructive process could not be inherited since the donor pancreas came from a non-diabetic co-twin, and the rapid destruction of apparently normal β-cells when transplanted from the non-diabetic to the diabetic twin indicated that the destructive process was recurrent recipient-mediated β-cell specific cytotoxicity. Furthermore, this cytotoxic memory was retained for at least 17 years.

The most characteristic pathological finding in the pancreases of IDDM patients at the onset of disease is the mononuclear cellular infiltration within the pancreatic islets, referred to as "insulitis" by Von Meyenburg in 1940 (7). Insulitis is accompanied by

the specific destruction of islet β-cells (8), and after approximately 90% of the β-cell mass has been destroyed the clinical manifestations of IDDM present themselves (9). However, the extent of insulitis within individual patients is very variable, affecting 13-75% of islets (10). MacLaren (11) pointed out that the pace of these events may well relate to age of onset and the underlying genetic heterogeneity of the individual. As in other endocrine tissues undergoing active autoimmune destruction, Gepts (9) reported the presence of lymphocytes, macrophages and plasma cells in pancreatic sections from IDDM patients who died soon after diagnosis. Furthermore, areas of pancreas containing islets devoid of β-cells and insulitis were often observed adjacent to pancreatic lobules containing many islets, predominantly composed of hyperactive β-cells (9). Some of these islets were free of insulitis, whilst others were infiltrated by a few lymphocytes or heavily infiltrated. Thus, biopsies taken from a " diabetic " pancreas showing patchy insulitis may give rise to inconsistent findings, and may explain why Doniach and Morgan failed to observe insulitis in any of 13 recent onset diabetic patients studied (12).

Conventional histological examination of fixed pancreatic tissues does not allow the precise characterisation of the various lymphocyte subpopulations infiltrating the islets. In 1985, Bottazzo et al (13) examined frozen blocks of a fresh pancreas obtained at post-mortem from a type I diabetic child who died shortly after diagnosis. Using various monoclonal antibodies (moAb) with single or double fluorochrome techniques, it was shown that most of the mononuclear cells in the inflammatory infiltrate were T lymphocytes. Cytotoxic/suppressor ($CD8^+$) lymphocytes formed the majority of T cells, but helper/inducer ($CD4^+$) T lymphocytes were also present. A large proportion of these $CD4^+$ T lymphocytes expressed HLA class II molecules, and a lesser number were positive for interleukin-2 receptors (IL-2R), demonstrating that infiltrating T cells were activated, and suggesting a specific immune response

directed against islet autoantigens. Many B lymphocytes were also present around individual islets, as were immunoglobulin G (IgG) deposits, thus demonstrating antibody penetration following injury to the islet cell membrane. The presence of macrophages in the inflammatory infiltrate, capable of engulfing β-cells, has also been reported in human diabetic pancreases (14). However, Foulis and Farquharson (15) noted a scarcity of macrophages in the islets of diabetic patients at the time of diagnosis, and hence could not attribute a myeloid/lymphoid phenotype for the class II positive cells observed in these islets. This was further substantiated using anti-class II and anti-macrophage antisera which identified macrophages primarily in the exocrine portion of the pancreas, and their appearance and distribution resembled that of tissue resident macrophages, having no apparent aggression towards islets.

1.1.2.3. IMMUNOPATHOGENESIS

1.1.2.3.1. Cellular immunity

Evidence accumulated over the last several decades strongly supports the hypothesis that IDDM has an autoimmune pathogenesis (16,17). Evidence includes an immunogenetic susceptibility, cell-mediated immune abnormalities detected in the peripheral blood, lymphocytic infiltration in islets of diabetic patients and autoantibodies to islet cells.

Extensive research has been carried out on the cell-mediated processes involved in the autoimmune destruction of islet β-cells. The leucocyte migration inhibition test using homogenate prepared from pooled porcine pancreas, has shown that peripheral blood leucocytes from newly diagnosed IDDM patients are sensitised to pancreatic antigens (18,19) and it is likely that these immunocytes recognise different pancreatic β-cell

antigens from those reacting with islet cell antibodies (ICA). In addition lymphocytes from IDDM patients have been shown to produce cytotoxicity to various islet cell targets *in vitro* (20), and Boitard et al (21) reported that mononuclear cells from 80-90% of IDDM patients specifically inhibited glucose-stimulated, but not arginine-stimulated, insulin release from mouse islets *in vitro* (22).

Conflicting results for total numbers of peripheral T and B lymphocytes have been reported in IDDM patients. These discrepancies may be partially explained by differences in the metabolic control of patients (23), including hyperglycaemia and ketoacidosis (24,25), and the analytical technique employed (26). However, both normal T and B lymphocyte numbers (27,28) and decreased numbers of circulating T lymphocytes (29) have been reported in IDDM patients irrespective of glycaemic control.

The use of moAb specific for lymphocyte subsets has failed to resolve this discrepancy, since both increased (30) and decreased (31) ratios of helper/suppressor cells have been reported in IDDM patients. With respect to lymphocyte subsets, most studies report that the frequency of helper/inducer cells are normal at onset of IDDM (31-33). Cytotoxic/suppressor T lymphocytes have been reported as normal (32,34), increased (27,31) and decreased (28,33). A reduction in cytotoxic/suppressor T cells would be consistent with one of the classic theories of the pathogenesis of autoimmune disease, namely that a defect in the suppressor mechanism allows latent autoimmunity to become frank autoaggression. Jackson et al (32) initially reported an increase in the number of activated T cells in the circulation of newly diagnosed IDDM patients. Approximately 10% of circulating T lymphocytes were MHC class II-positive in 9 of 11 diabetic patients, compared with 1% class II-positive T cells in controls. Several studies have since confirmed these

findings (27,31,34-36). De Berardinis et al (36) further described the presence of IL-2R-bearing, i.e. activated, T cells in approximately 50% of recently diagnosed IDDM patients, and most of these activated T cells were of the helper/inducer ($CD4^+$) subset.

Diminished activity and functional abnormalities of suppressor T cells have been reported in IDDM. Using antigen non-specific assays, several reports have described an impairment of suppressor T lymphocyte function in diabetic patients at, or close to, the time of diagnosis (26). However, interpretation of these results is difficult since T cell functions are generally grossly altered in diabetic individuals, primarily due to abnormal metabolic status (23). This conclusion is supported by the reversibility of the defect in most cases upon the restoration of metabolic control by administration of insulin (37). However, abnormal suppressor T cell function has also been demonstrated in non-diabetic HLA-DR3 individuals (38) and in approximately 20% of healthy first degree relatives of IDDM patients (39), suggesting that abnormal suppressor T cell function may alternatively be related to genetic background. A decrease in the number of natural killer (NK) cells circulating in newly-diagnosed IDDM patients has also been reported, but this appears to be a genetically determined abnormality which may be compensated by the observed increase in NK activity per cell, although diminished activity has also been reported (40). K cell activity is increased (41).

The role of two isoforms of the cell surface marker CD45, which divide T lymphocytes into " naive " and " memory " populations, has recently been reviewed (42). Naive or virgin lymphocytes bear the CD45RA isoform of CD45, are relatively unresponsive to antigen stimulation, have a suppressive influence on other lymphocyte functions, and form the vast majority of lymphocytes in the foetal circulation. In

contrast, memory or primed cells bear the CD45RO isoform, proliferate in response to antigen, promote a range of functions in other lymphocytes and are increasingly represented in peripheral blood from birth onwards. The balance of these two populations is interpreted as having an overall influence on immune functions, with imbalances resulting in either predominantly suppressive or promotive activity.

Interest in the naive and memory populations in IDDM patients stems from three seperate studies which have produced a clear and consistent picture in which there is a preponderance of $CD45RA^{+}CD4^{+}$ lymphocytes at diagnosis of disease. Later studies extended these findings, demonstrating that $CD45RA^{+}CD8^{+}$ lymphocytes were also overexpressed. In addition, using a technique to label naive and memory markers simultaneously, it was demonstrated that the increase in naive lymphocytes at diagnosis of IDDM is a result of an increased number of CD4 and CD8 lymphocytes simultaneously co-expressing both CD45RA and CD45RO. These double-positive lymphocytes are in transition between the two functional states, but as yet the reason for an expansion in these cells in association with the development of IDDM remains unclear.

The studies described here strongly support a role for cell-mediated immunity in the pathogenesis of IDDM. However, as the transfer of diabetes using peripheral blood lymphocytes from an IDDM patient is unsuccessful, the requirement of an initial triggering stimulus prior to activation of autoreactive lymphocytes appears necessary. As discussed earlier, genetic influences are important in the development of IDDM but the disease must be, at least in part, determined by non-genetic factors, including non-MHC genes and environmental factors, that act in a genetically susceptible individual.

1.1.2.4. GENETICS

Many familial studies on IDDM have been published, more recently by Tillil and Kobberling (43), and all studies show that diabetes occurs more frequently in families with a history of diabetes than would be expected by chance alone. A risk of IDDM among first-degree relatives of approximately 5% has consistently been reported, corresponding to a risk 10-20 times higher than that in the general population, although IDDM is more likely in the progeny of diabetic fathers than diabetic mothers (44,45). The risk of diabetes in the sibling of a diabetic child is related to the number of haplotypes the sibling shares with the diabetic proband (46) unless the shared haplotype is the heterozygous HLA-DR3/4, when the absolute risk of IDDM is 25% (47). In addition, second-degree relatives show an increased risk, but this risk is considerably less than that of first-degree relatives.

Twin studies provide a unique tool in genetic research, and by comparing the frequency of concordance (both members of the twin pair affected) of monozygotic (identical) twins with that of dizygotic (fraternal) twins, an estimate of the heritability may be obtained. Monozygotic twins share all genes and in theory should be concordant for those disorders with pure genetic aetiology. Dizygotic twins share only half their genes and thus are no more alike genetically than a pair of siblings. Barnett et al (48) demonstrated that the estimated concordance rate, i.e. the risk of developing IDDM in monozygotic co-twins was 30-50%, and the heterozygous HLA-DR3/4 phenotype was more prevalent in concordant pairs (59%) than with discordant pairs (28%) (49). Olmos et al (50) reported a concordance estimate of approximately 36%, which is probably a more accurate estimate. Taken together, twin and familial studies provide strong evidence that genetic factors play an

important role in the susceptibility of individuals to IDDM, and indicate the requirement of other factors to elicit the disease in genetically susceptible individuals.

Following the initial detection of the association between markers for the class I HLA-B locus, namely HLA-B8 and B15, and IDDM in the early 1970's (51), a large number of association studies have been reported (52). However, with the development of cellular typing for class II molecules and the isolation of class II antisera, it became clear that HLA-B markers appeared to be secondary to stronger population associations of IDDM with class II HLA-DR markers (53,54), particularly DR3 and DR4 which have consistently been positively associated with diabetes. This implies that the associations of class I antigens are probably due to linkage disequilibrium between class I and class II genes, resulting in high frequencies of certain combinations of alleles or haplotypes in the population. Indeed, over 95% of Caucasians with IDDM have the DR3 or DR4 antigens (allelic products of the class II HLA-DRβ1 and DRα loci respectively) or both, compared with approximately 50% of controls (52) demonstrating the increased risk for HLA-DR3/4 heterozygotes (55,56). This risk is greater than the sum of the risks for HLA-DR3 and DR4 homozygotes. These findings have led to the hypothesis that two different genes, one linked to HLA-DR3 and one linked to HLA-DR4, may each confer susceptibility (49). The DR4 haplotype is more common in younger diabetic individuals and is associated with a more rapid loss of β-cell mass and a short " honeymoon " period. In contrast, diabetic patients expressing DR3 usually show a slower disease course, with a prolonged " honeymoon " phase. HLA-DR2 is negatively associated with IDDM in Caucasian populations (57), and is only observed in 2% of patients with IDDM compared with a frequency of 30% in the normal population (58). The relative risk of IDDM in HLA-DR2 individuals is approximately 75 times less than that of the HLA-DR3/4 heterozygotes (59).

The class II gene products are encoded by loci in the HLA-D region of the MHC and consist of an α-chain (32 kD) and a β-chain (29 kD) which form a heterodimer on the surface of cell subsets including macrophages, B and activated T lymphocytes and dendritic cells (60). Three related and highly polymorphic class II antigens have been identified, designated HLA-DR, DQ and DP, and the polymorphic residues have been postulated to interact with the T cell receptor and/or foreign antigen (61,62). The recognition of foreign antigen peptide fragments associated with an MHC class II molecule leads to T cell activation. The haplotypes defined by class II serologic typing, i.e. the HLA-DR and DQ serotypes, are genetically heterogeneous and were initially subdivided using serological and cellular typing analysis. Using these techniques, the HLA-DR4 locus could be subdivided into five different subtypes (63), two of which, namely Dw10 and Dw4, are increased in HLA-DR4$^+$ IDDM patients over HLA-DR4$^+$ controls (64,65). Evidence suggests that variation in the DQβ region may be more strongly associated with risk for IDDM than variation in the DR region, at least regarding the HLA-DR4 associated susceptibility. Several groups have reported a variation in the DQβ region in linkage disequilibrium with DR4, which occurs with increased frequency in IDDM patients. This variant, formerly called the DQw3.2 allele and now called DQw8, occurs in as many as 90-95% of those IDDM patients who carry the HLA-DR4 allele (66) and in approximately 60-75% of DR4-carrying non-diabetic controls (52). In contrast, the DQw3.1 allele in the DQβ region, now called DQw7, is decreased.

Restriction fragment length polymorphism (RFLP) analysis studies have demonstrated that linkage disequilibrium is very strong between the HLA-DR and DQ subregions. Owerbach et al (67) were the first to discover a HLA-DQβ1 DNA polymorphism using a HLA-DQβ gene probe and the restriction enzyme Bam H1. This group found a 3.7 kB DQβ gene related fragment that was present in 2% of

Danish IDDM patients but as many as 37% of racially matched controls. These findings have consequently been extended by many groups, and the overall conclusion confirms that the diabetogenic gene(s) may lie very close to the HLA-DQ locus (68-71). Other DQβ chain RFLP have been identified, and these DQβ associations overlap other HLA-DR specificities, such as HLA-DR1, which are associated with IDDM.

Class II A and B chain sequence data utilising the polymerase chain reaction method showed that, disappointingly, the first domain of the DQw8 sequence did not differ between IDDM patients and non-diabetic individuals (72,73). Sequencing the polymorphic first domain of DRβ, DQα and DQβ revealed no unique class II sequence found exclusively in IDDM patients, indicating that HLA-conferred susceptibility to IDDM is not mediated by structurally distinct HLA class II molecules (72). This group (72) further suggested that the genetic susceptibility of IDDM associated with genes of the HLA-DQ locus may be explained by amino acid variation within the DQβ molecule. Comparison of the amino acid sequence of the positively and negatively IDDM associated HLA-DQw8 and DQw7 alleles respectively revealed only six amino acid differences between the two alleles, four of which were in the N-terminal polymorphic domain at residues 13, 26, 45 and 57. Coding sequences of DQw8 and DQw7 were identical between controls and patients. Accordingly, these four amino acids might be responsible for the associations of these two haplotypes with IDDM. Todd et al (72) cloned and sequenced HLA-DQβ genes in HLA-DR homozygous typing cells possessing susceptibility haplotypes, i.e. DR4-DQw8, DR3-DQw2, DR1-DQw1.1 and DR3-DQw1.AZH, and haplotypes protective or neutral to the disease, i.e. DR4-DQw7, DR5-DQw7, DR2-DQw1.12 and DR2-DQw1.2. In the latter group an aspartate was found at position 57 of the DQβ molecule in all haplotypes whereas in all haplotypes associated with IDDM

susceptibility, a different amino acid was found, often serine, valine or alanine. No other polymorphic residues within the sequenced domains of either DRβ, DQα and DQβ showed a similar association. According to this hypothesis, IDDM develops in individuals carrying two non-Asp-57 DQβ alleles, as is the case for DR3/4 IDDM patients. However, this is not only observed in DQβ chains associated with DR3 and DR4, as the IDDM-protective DR2-associated DQβ chains contain DQβ Asp-57, whereas 77% of IDDM patients with DR2 carry the DQw1.AZH allele with serine at position 57 (72).

A possible mechanism by which this observation might be of importance has come from the recent understanding of the three-dimensional structure of the HLA molecule. The presence of a charged amino acid, e.g. aspartic acid, at residue 57 of the DQβ chain may allow the formation of a salt bridge with a conserved arginine at residue 79 in the DQα chain (74). This salt bridge would be situated in one end of the foreign antigen binding cleft and may therefore affect antigen binding capacity or T cell interaction of the HLA-DQ molecule. In contrast, a non-charged residue, e.g. serine, valine or alanine, at position 57 would prevent the formation of the salt bridge.

However, the concept of this single HLA-DQ residue at position 57 as being totally responsible for HLA-linked IDDM is incompatible with currently available data, and there are a number of exceptions to this association (75). Firstly, many more normal individuals have been shown to possess this same characteristic, thus decreasing the disease specificity of this finding. Indeed, calculations of the absolute risk for IDDM suggest that this molecular observation increases the specificity only modestly, i.e. at most two-fold. Secondly, aspartic acid in position 57 is not always associated with resistance to IDDM, particularly in Japanese type I diabetics where as many as 24%

of the IDDM patients appear to be homozygous for aspartate at position 57 of the DQβ chain (76,77).

Owerbach et al (78) found that other amino acid residues (other than residue 57 of the DQβ chain) are important for IDDM susceptibility in Caucasians, and the DQα gene also has a role in diabetes susceptibility. In support of the concept of additional HLA-D locus determinants, Sheehy et al (65) have provided data that implicate both DR and DQ simultaneously.

In conclusion, the mode of inheritance for IDDM remains unknown but is clearly polygenic (72). The weight of evidence indicates that the DQα1 and DQβ1 loci both exert an influence on predisposition to IDDM. Combined analyses of twin and family data suggest that there are at least two HLA-linked susceptibility loci : the HLA-DR4-associated determinant in the absence of HLA-DR3 is the most diabetogenic and is inherited in a dominant/intermediate fashion (56,79). Conversely, DR3-associated susceptibility in the absence of HLA-DR4 appears to be recessive (56). However, mere possession of these haplotypes is not sufficient for the development of IDDM, and no abnormalities present exclusively in class II genes of IDDM patients have been identified. Furthermore, the increased risk of the DR3/4 heterozygote is clearly not explained solely by the DQβ non-Asp hypothesis. Therefore, the more specific diabetes-predisposing genes or combination of genes that are carried on HLA haplotypes within IDDM families still need to be identified.

1.1.2.5. NON-MHC GENES

At least four non-MHC loci have been associated with susceptibility to IDDM, namely the Kidd blood group on chromosome 18 (80,81), immunoglobulin heavy

chain haplotypes on chromosome 14 (79), the T cell receptor β-chain on chromosome 7 (82,83) and small inserts of the DNA variable region proximal to the insulin gene on the short arm of chromosome 11 (84). The involvement of the insulin gene is further supported by studies of the extended haplotype of this region (85), and by the observation that this gene provides susceptibility to IDDM regardless of HLA type (86). However, the effect of this locus is much smaller than that of HLA, which provides the major genetic susceptibility to IDDM.

1.1.2.6. ENVIRONMENTAL FACTORS

Environmental factors might function as initiating factors which begin or continue the aetiologic processes that eventually lead to IDDM, or as precipitating factors which convert pre-clinical diabetes into clinical disease. Evidence of the aetiological importance of environmental factors include the increase in IDDM incidence with time (87), the geographical variation in IDDM within and between countries (88) and the well-documented seasonal variation of the clinical onset of IDDM, with relatively more cases diagnosed during the autumn and winter months (89,90). However, no individual environmental determinants of IDDM have been detected with certainty, although viruses (91,92), dietary factors (93,94), socio-economic standard (95) and stressful life events (96) have been implicated.

1.1.2.6.1. Viral infection

The most likely candidates for exogenous agents contributing to IDDM appear to be viruses. Several studies have suggested that viruses play an important role in the pathogenesis of diabetes on the basis of (a) the presence of viral-specific antigens in the pancreatic islets and destruction of β-cells of diabetic patients (97), (b) the

presence of viral antibodies with rising titres in paired sera from newly diagnosed IDDM patients (97,98), (c) a high frequency of Coxsackie B virus-specific IgM antibody in newly identified diabetic children (99), (d) β-cell damage in children who died of well-documented overwhelming viral infections (101), (e) the isolation of viruses from patients with acute-onset diabetes and the demonstration that these isolated viruses could induce diabetes in mice (97,98), and (f) the association of autoantibody production with certain viral infections, including congenital rubella and persistent cytomegalovirus infection (101,102). Diabetes is frequently found in patients with congenital rubella syndrome, although disease may take 5-20 years to develop (103), and these individuals have a significantly increased frequency of HLA-DR3 and a significantly decreased frequency of HLA-DR2 (97). The seasonality of the onset of diabetes (104) and the increased viral antibody titres at this time (99) provide further evidence that a viral infection may precipitate disease. It has been hypothesised that viruses may be responsible for the inappropriate class II expression observed on the surface of islet β-cells of diabetic patients that are undergoing autoimmune attack (105). In these islets, glucagon-secreting α-cells, somatostatin-secreting δ-cells and exocrine cells do not express class II antigens, which is consistent with the sparing of these cells in the killing process. Once β-cells become positive for class II molecules, they themselves could act as antigen presenting cells (APC) of its own surface autoantigens to T cells (106), thus by-passing the requirement for conventional APC such as macrophages and dendritic cells. If this phenomenon occurs in genetically susceptible individuals, i.e. those with the predisposing HLA-DR3 and/or DR4 haplotype, but not HLA-DR2-positive individuals, autoantigens normally expressed on the surface of β-cells can be presented to helper T lymphocytes, leading to their activation. Subsequently, effector B and cytotoxic T cells are stimulated. The outcome of the induction of autoimmune T cells may depend upon the activity of regulatory mechanisms, such as suppressor T

cell activity, which normally downregulates self-responsiveness (107), and production of anti-idiotypic antibodies, i.e. antibodies directed against particular clones of autoreactive lymphocytes (108). Interferon-γ (IFN-γ) and tumour necrosis factor (TNF-α) produced by invading lymphocytes and macrophages are likely to have a role in perpetuating the class II hyperexpression (109), although a more direct cytopathic role for interleukin-1 (IL-1) has been suggested due to its cytotoxicity to β-cells *in vitro* (110).

Enhanced HLA class I expression is another phenomenon observed in the islets of IDDM patients, which may explain the abundant presence of cytotoxic T cells among infiltrating lymphocytes at the time of diagnosis of diabetes (111). These T cells appear to be the main effectors in the final selective destruction of β-cells. However, this hypotheses is only one of several proposed over the years to explain how the autoimmune attack against pancreatic β-cells is initiated in the IDDM patient. Hopefully, amalgamation of individual theories will elucidate the pathogenesis of diabetes.

1.1.2.6.2. Diet

The autoimmune process leading to IDDM could be precipitated by a factor present in the diet during infancy (112). Cow's milk in particular has been proposed as a possible trigger (113). Animal studies have suggested bovine serum albumin (BSA) is the milk protein responsible and an albumin peptide containing 17 amino acids (ABBOS) may be the reactive epitope. Antibodies to this peptide react with p69 which is a β-cell surface protein that may represent the target antigen for milk-induced β-cell-specific immunity. All diabetic patients had increased serum concentrations of IgG anti-BSA antibodies (but not of antibodies to other milk

proteins), the bulk of which were specific for ABBOS. IgA antibody levels were also elevated. Concentrations of these antibodies decreased after diagnosis and reached normal levels in most patients within 1-2 years. Savilahti et al (114) investigated the humoral response to cow's milk and β-lactoglobulin in paediatric patients with newly diagnosed IDDM by enzyme-linked immunosorbent assay (ELISA). Patients <3 years of age had a markedly higher median level of IgG and IgA antibodies to cow's milk and IgG antibodies to β-lactoglobulin compared with control subjects. Older diabetic patients (3-15 years of age) also showed higher levels of IgA antibodies to cow's milk and β-lactoglobulin than age-matched controls or non-diabetic siblings, although levels were lower than those found in the younger group. These results suggest that the proteins of cow's milk may trigger the autoimmune process of IDDM.

1.1.3. CONCLUSION

IDDM is a group of disorders that are the result of destruction of the insulin-producing β-cells of the pancreas, principally by autoimmune-mediated mechanisms. It is possible that some cases are due to direct viral destruction of the β-cells but, if so, these would appear to be only a small fraction of the total. In the majority of cases, this autoimmune destruction of the β-cells proceeds over months and years. The major defined risk factor for IDDM is genetic susceptibility, and at least two major genes have been localised to the HLA complex on chromosome 6, one associated with DR3, the other principally with DR4. When occurring together, these two genes dramatically increase the susceptibility to IDDM. There is tentative evidence for a smaller contribution by other genes, such as the DNA polymorphic region proximal to the insulin gene on chromosome 11. Since human IDDM has a long natural prediabetic period, if both the detailed immunologic

processes and the exact genetic susceptibility could be identified, primary disease prevention might become possible.

1.2. **PRIMARY PREVENTION OF IDDM**

Primary prevention not only depends on elucidating the mechanism of β-cell destruction but also the ability to accurately predict individuals who will develop clinical IDDM, as distinct from identifying subjects with increased susceptibility to disease or with insulitis who never progress to metabolic decompensation. Current predictive markers used to determine individuals at high risk of developing type I diabetes include HLA-screening and autoantibodies directed against components of the islet cell, which are observed in the circulation well before the clinical onset of diabetes.

1.2.1. HLA-SCREENING

As mentioned earlier, it has been estimated that 60% or more of the overall genetic susceptibility to IDDM is contributed by HLA-region genes. Thus, an accurate marker for the HLA-linked genetic susceptibility would identify the majority of those individuals in the population who are genetically at increased risk of developing IDDM. However, even the identical co-twin of a diabetic individual, who is genetically at the highest risk for diabetes, has only a 30-40% chance of developing diabetes.

There are two ways in which HLA genes might be used in screening : (a) to test for " high-risk " HLA types randomly in the population, and (b) to test for genetic risk in siblings, offspring and other relatives of IDDM patients. Approximately 50% of the

non-diabetic population have the same DR types as patients with IDDM. Thus, at least 98% of the people with HLA-DR3 or DR4 will never develop IDDM. For every 1000 persons with HLA-DR3 or DR4 in the population, only 2-4 will develop IDDM in their lifetime. HLA typing will therefore result in many more false positives than true positives in terms of genetic risk.

After identical twins, the individuals at highest risk of developing IDDM are the siblings of type I diabetic patients, who have an overall 5-10% risk for developing IDDM. HLA testing can be used to define these risks within families (56,115,116). A sibling who shares all HLA types with his or her diabetic sibling has a 12-24% risk of developing IDDM, a sibling with one haplotype in common has a 4-7% risk, and a sibling who shares no HLA types has a 1-2% risk.

HLA typing is probably best used as a research tool to elucidate the development of diabetes, whereas autoantibodies to islet cell components might be used to screen for preclinical IDDM in HLA-susceptible individuals, either within families or in the general population (11,16).

1.2.2 AUTOANTIBODIES

Evidence accumulated over the past two decades describing the detection of autoantibodies directed towards pancreatic islets in the sera of diabetic patients has further supported the view that IDDM is an autoimmune disease. Only those autoantibodies showing sufficient diabetes specificity to be used as markers of an ongoing autoimmune process against pancreatic β-cells will be discussed further.

1.2.2.1. Islet cell cytoplasmic antibodies

Islet cell cytoplasmic antibodies (ICCA) were first described in type I diabetic patients who had other co-existent autoimmune polyendocrine disorders (117) and subsequently in 60-80% of IDDM patients shortly after diagnosis (118). In contrast, only 2-4% of healthy first degree relatives of patients with IDDM and 0.2% of the general population are positive for ICCA (119). ICCA decreased both in frequency and titre in the circulation of diabetic patients after disease onset, falling to 20%, 2-5 years after diagnosis (120,121). This decrease may reflect a gradual loss of antigenic stimulus as islets are destroyed, leading to an absolute absence of β-cells (122). ICCA reacting with islet cytoplasmic cell antigens were detected by indirect immunofluorescence in thin cryostat sections of human pancreas, using tissue from blood group " O " donors, thus preventing interference with isoagglutinins (123). The autoantibody was shown to be exclusively of the IgG subclass, and predominantly IgG1 (124). However, despite 20 years of research the target antigen of ICCA has still to be fully characterised, although studies strongly suggest a ganglioside as target (125).

ICCA lack specificity for the β-cell since they can also react with the cytoplasm of the other islet cell types, namely α- and δ- cells, suggesting that these endocrine cells share a common autoantigen (126). Some ICCA, in particular those of high titre, are able to fix complement (127), the major effector of damage in humoral immunity, and later studies have shown a definite correlation between titre and complement-fixing ability of ICCA (124,128). Complement-fixing ICCA are present in fewer patients than conventional ICCA, though these autoantibodies are more strongly predictive of IDDM (129).

Islet cell specific autoantibodies are thought to mediate islet cell injury by two mechanisms. The first involves complement-dependent antibody-mediated cell mediated cytotoxicity (CAMC), where antibodies bound to a cell surface antigen link to and activate the complement pathway leading to increased vascular permeability, chemotaxis for phagocytes and eventual cell lysis. These events lead to the activation of the second mechanism, i.e. antibody-dependent cellular cytotoxicity (ADCC). Cross-linking of Fc antibody receptors on the surface of macrophages induces phagocytosis and islet cell damage, probably due to the secretion of lymphokines.

Although detectable several years prior to the onset of IDDM, ICCA are unlikely to mediate CAMC or ADCC, and thereby β-cell cytotoxicity in IDDM patients, since they are directed to cytoplasmic components and lack β-cell specificity. Also, transfusion of ICCA-positive sera does not transfer diabetes to animals, and no infant born to an IDDM mother has been reported to have aquired the disease in utero, even though maternal antibodies readily pass through the placenta to the foetus (130).

1.2.2.2. Islet cell surface antibodies

Islet cell surface antibodies (ICSA) are by definition autoantibodies which react with surface antigens on viable islet cells, and were initially discovered in 1975 by MacLaren and co-workers who found antibodies reactive with the surface of cultured human insulinoma cells in approximately 87% of diabetic patients (131). Subsequently, indirect immunofluorescence was used to detect the presence of ICSA in the sera of newly diagnosed IDDM patients (132,133). ^{125}I-labelled second antibodies (134) and ^{125}I-labelled protein A (135) have also been used for determining ICSA .

The prevalence of ICSA has been shown to vary widely among studies. When dispersed rat or mouse islet cells are used, frequencies of 30-60% have been reported among IDDM patients (132,133). It should be noted that human sera should be preabsorbed with rodent tissues to diminish non-islet-specific cross-reactions between human antibodies and the rat islet cells under these conditions. Since ICSA is not species specific, the sensitivity of ICSA detection may be higher when human islet cells are used instead of rodent islets. Indeed, 82% of newly diagnosed IDDM patients were positive for ICSA in a study using cultured neonatal human islets (136).

In contrast to ICCA, ICSA appear to be exclusively β-cell specific, particularly in patients aged under 30 years (133,137-139). However, in older diabetic patients, ICSA were occasionally preferentially bound to α- and polypeptide cells. In addition, ICSA have also been detected in patients with non-insulin-dependent diabetes mellitus (NIDDM) (139), in 30% of non-diabetic patients suffering with thyroid disease (140), and in animal models of spontaneous diabetes, namely the BB rat (141) and non-obese diabetic (NOD) mouse (142). Again, as for ICCA, the prevalence of ICSA decreases with an increasing duration of IDDM (132).

An important feature of ICSA is its ability to lyse cultured islets in the presence of complement (133,137,138,143), and this ICSA-complement-dependent cytotoxicity was preferentially directed against β-cells (133,137), although approximately 10% of non-β-cells were also lysed (137). ICSA are thought to mediate their cytotoxicity via ADCC in which ICSA bind to Fc receptors on cells such as macrophages and NK cells.

ICSA are more likely to mediate CAMC or ADCC than ICCA, as they react with antigens on the surface of islet cells. Indeed, ICSA-positive sera have been reported to mediate CAMC using rat and hamster islet cells and rat insulinoma cells as target (133,143,144). However, both ICCA and ICSA are present in healthy subjects without causing disease, and neither positively segregates with the diabetogenic haplotype in families (144). These autoantibodies must therefore be considered of limited value as predictive markers of IDDM.

1.2.2.3. Insulin autoantibodies

Spontaneous autoantibodies to insulin (IAA) were initially described in 1970 in the hypoglycaemic syndrome, which was later termed the insulin autoimmune syndrome (145). IAA were subsequently identified in untreated newly diagnosed diabetic patients (146), their first degree relatives (147,148) and patients with other unrelated autoimmune disorders (149). More recently, circulating IAA have been found to be particularly prevalent in young diabetics at diagnosis, although frequencies reported vary between different groups. This variation is most likely due to the use of different assay systems, i.e. radioimmunoassay (RIA) versus ELISA, and the use of different insulin species as substrate, i.e. human versus bovine or porcine insulin. Palmer (150), using a liquid phase RIA and a porcine insulin tracer, detected IAA in 18% of children at diagnosis of diabetes, but siblings of the diabetic probands appeared negative for IAA. Other studies have described a frequency of IAA closer to 40% (151), whereas a frequency of only 3.8% was detected in a study of 26 adults at diagnosis of IDDM (152). Controvesy also exists between the correlation of IAA with ICCA, with some groups reporting an association (147,153) and others not (152,154). Despite assay differences, IAA can be detected as early as ICCA and ICSA in the prediabetic period, but carry minimal pathogenic potential.

IAA assays also detect antibodies reacting with proinsulin as well as insulin, although autoantibodies reacting specifically with proinsulin have been detected in 14% of IAA-negative patients (155).

1.2.2.4. Glutamic acid decarboxylase autoantibodies

Autoantibodies to a 64 kD protein were first discovered in the early 1980's by Baekkeskov et al (156). Crude detergent lysates of ^{35}S methionine-labelled human islets were immunoprecipitated using sera from newly diagnosed diabetic children and sera from healthy control individuals. Eight of ten children immunoprecipitated a 64 kD protein, whereas none of the control sera did. This 64 kD protein is found exclusively in the plasma membrane of β-cells, comprising at most 0.01% of total trichloracetic acid precipitable counts in ^{35}S methionine-labelled human islets. The high affinity and specificity of autoantibodies to the 64 kD protein, recently identified as glutamic acid decarboxylase (GAD), are demonstrated by their ability to bind to and specifically precipitate this protein in the presence of a huge excess of islet proteins (157).

The prevalence of GAD autoantibodies at clinical onset of diabetes is high, with 81% of 134 newly diagnosed IDDM patients being positive compared with 2% of 111 healthy controls (156,158). Additionally, these autoantibodies were not detected in the sera of patients suffering from Hashimoto's thyroiditis, Graves' disease and systemic lupus erythrematosus, demonstrating their specificity for the β-cell.

In the first familial study of 14 prediabetic individuals, followed from 4-91 months prior to IDDM onset, GAD autoantibodies were detected up to 8 years before clinical onset in 11 patients (159). These findings have been verified by Atkinson et al

(158), who detected GAD autoantibodies in the first serum sample in 23 of 28 individuals, 2-75 months prior to IDDM onset. Interestingly, of the 23 GAD antibody-positive patients, ten and five were negative for IAA and ICCA respectively. However, it should be noted that as the majority of β-cells disappear prior to the clinical onset of diabetes, the prevalence of GAD autoantibodies, ICCA and ICSA may decrease also. Thus GAD antibody-negative individuals at onset may have had circulating antibodies during the prediabetic period. For these reasons MacLaren (11) suggested that the incidence of autoantibodies may even be higher if the sera of patients is analysed regularly. The progress of GAD autoantibodies in positive patients after the onset of IDDM suggest that GAD antibody immunoreactivity declines slowly and may take several years, despite a very low concentration of the protein.

More recently, cloning and sequencing studies revealed the existence of two GAD isoforms, GAD_{65} and GAD_{67}, which share 67% sequence homology and are encoded by seperate genes on different chromosomes (160). GAD_{65} has been shown to be identical to the 64 kD autoantigen, and is the main target for GAD autoantibodies in sera from recent onset IDDM patients (161).

The autoantibody directed against GAD may be the most promising potential marker for prediabetes. It is β-cell specific and appears before ICSA and IAA, i.e. many years beore the clinical onset of IDDM. The observation that autoantibodies against the 64 kD protein appear before insulitis and other immune abnormalities in both the BB rat (162) and NOD mouse (163) suggests that the antibodies may be primary rather than secondary to β-cell destruction. Furthermore, Petersen et al (164) have recently developed a simple, reproducible and quantitative immunoprecipitation radioligand assay that allows large numbers of serum samples to be tested for GAD_{65}

antibodies in a relatively short time, thus allowing screening of individuals with or without a family history of IDDM for the presence of this marker. However, it should be noted that none of the autoantibodies are absolute predictive markers.

More recently, the risk of progression to IDDM has been assessed using " decision tree " analysis (165) which can be applied to individuals with or without a family history of IDDM. Indeed, it is possible to predict IDDM with a high degree of specificity in a small subset of first-degree relatives of IDDM patients. However, as approximately 90% of future cases of IDDM will come from individuals who have no close relative with IDDM, prediction within the general population must be a main priority for the future.

1.2.3. CONCLUSION

Predicting the development of IDDM in an individual subject is difficult and not wholly accurate, therefore population screening for IDDM at this time is unrealistic. However, screening of first degree relatives might be justified if an effective and safe method of preventing type I diabetes was available. As mentioned, primary prevention depends on the elucidation of the precise sequence of events leading to mass destruction of islet β-cells, which, because of the inaccessibility of the human pancreas as target organ, depends in turn on the availability of an appropriate animal model of diabetes. These models can also be used to investigate the role of environmental factors in the clinical expression of diabetes and assess intervention studies.

1.3. ANIMAL MODELS OF IDDM

Many models of either spontaneous or chemically-induced insulin-dependent diabetes mellitus (IDDM) have been described (166). In this thesis, studies performed on one particular animal model will be reported : the spontaneously diabetic BB rat. In addition, references will be made to two other commonly used animal models of IDDM, namely the NOD mouse and the streptozotocin (STZ)-induced diabetic mouse.

1.3.1. THE SPONTANEOUSLY DIABETIC, INSULIN-DEPENDENT BB RAT

1.3.1.1. INTRODUCTION

The BB rat was discovered in 1974 by the Chappel brothers in a commercial colony of albino Wistar derived rats at the Bio-Breeding laboratories (hence the designation BB) in Ottawa, Canada (167). Its appearance in the colony is thought to have been the result of a spontaneous mutation. Initially the cumulative incidence of diabetes in the colony was approximately 10% (165), but selective breeding has increased this figure substantially (168-171).

In 1977, a breeding colony of BB rats was established at the Animal Resources Division of Health and Welfare in Canada, which subsequently supplied the animals used to establish other breeding colonies, including our own in Edinburgh. In accordance with the recommended nomenclature, the original Ottawa colony, from which all BB rats descend, is designated BB, the colony at Worcester BB/W, that at Edinburgh BB/E, and so on (172). Prins et al (173) recently investigated the extent of the heterogeneity among 26 distinct lines of BB rat and reported considerable

variability, suggesting that the genetics of each line should be considered when comparing results obtained using animals from different colonies. The incidence of diabetes in diabetes prone (DP) BB rats not only varies widely from one colony to another, but also within colonies maintained under apparently constant conditions. This may be due to inadvertent changes in the environment. Environmental factors identified as affecting the incidence of diabetes include infection (174), diet (175) and stress (176). Undoubtedly other contributing factors have yet to be identified.

Particularly attractive features of this animal as a research tool for work in IDDM include a prediabetic period of approximately 2-3 months, involvement of both genetic and immune factors in aetiology, the fact that not all animals in litters with a high susceptibility to diabetes develop clinically overt IDDM, the absence of obesity, and the occurrence of functional and structural changes in the nerves, retina and kidneys of established diabetic animals. In addition, genetic manipulation and long-term selective breeding have led to an inbred line of diabetes-resistant (DR) BB rats which can serve as experimental controls, in order to determine whether experimental differences are diabetes or strain-related. Fewer than 1% of DR-BB in the Worcester colony have become diabetic through >32 generations of inbreeding (177,178). However, it should be emphasised that it is inappropriate to use non-diabetic littermates of animals with IDDM as controls, as many of these animals have impaired glucose tolerance, and are metabolically abnormal in other respects, despite not requiring insulin treatment for survival.

1.3.1.2. PATHOGENESIS

1.3.1.2.1. Clinical features

The onset of IDDM in the DP-BB rat is abrupt and acute, and has a peak incidence around the age of sexual maturation, i.e. between 60 and 120 days (170,171). The disease affects both sexes equally (169) and shows all the clinical features characteristic of the human disorder including polyuria, polydipsia, severe weight loss despite hyperphagia, and lethargy. Metabolically, BB rat and human diabetes are also analogous in many ways (179). At onset of IDDM, hyperglycaemia is associated with hypoinsulinaemia, hyperglucagonaemia, hyperketonaemia, hyperlipidaemia (including free fatty acids, glycerol, triglycerides and cholesterol) and uraemia. Blood levels of branched-chain amino acids and taurine, glycine, tyrosine, phenylalanine, ornithine and lysine are all elevated, whilst alanine and lactate are low (180). Increased urinary levels of nitrogen and 3-methylhistidine, together with enhanced ammoniagenesis, reflect a generalised catabolic state. Insulin responses to feeding and intravenous (i.v.) glucose challenge may be normal up to 10 days before disease onset (181). Death invariably ensues in the absence of exogenous insulin treatment.

1.3.1.2.2. Complications

Secondary systemic changes suggestive of human diabetic complications are found in long term insulin-treated diabetic BB rats. The abnormalities include alterations in nerve conduction and morphology, renal physiology and biochemistry, retina, hepatic metabolism, including accelerated gluconeogenesis and impaired glucose utilisation (182,183), gonadal dysfunction related to low circulating testosterone levels (184),

and cardiac dysfunction, associated with abnormalities in cardiac contractile proteins (185,186). Lymphocytic infiltration of the thyroid has been described in human IDDM and in both both diabetic and non-diabetic DP-BB rats. However, in the BB rat, thyroiditis is mild and the lesion does not progress to frank hypothyroidism (187), although the incidence of thyroiditis can be increased by the administration of dietary iodide (188). Atherosclerosis and severe microangiopathy, both of which are common in human IDDM, have not been reported in the diabetic BB rat (189).

Thus, many of the features of diabetes observed in the BB rat make it clinically analogous to human IDDM. Indeed, if human diabetes is closely related to diabetes in the BB rat, then much of the evidence obtained from studies involving this animal model will further substantiate and propagate the involvement of autoimmunity in the aetiology of IDDM. The most important of the secondary complications observed in the diabetic BB rat will be further discussed here.

1.3.1.2.2.1. Neuropathy

Diabetic neuropathy is the most common complication of diabetes. The BB rat develops reproducible structural changes in somatic peripheral and autonomic nerves which are characteristic of human IDDM, making this an ideal animal model in which to study the pathogenesis of neuropathy (190).

The first characteristic morphological change in the somatic nerves of the BB rat is observed within three weeks of diabetes onset, and consists of localised swellings of large axons at the nodes of Ranvier. This is associated with, and probably caused by, a 4-5-fold increase in intra-axonal sodium content resulting from a *myo*-inositol-related decrease in sodium/potassium ATPase activity. This intracellular

sodium accumulation also leads to the inactivation of sodium ion channels in the diabetic rat nerve, resulting in a decrease in maximum available sodium permeability (191). These metabolic abnormalities underly the functional neuronal changes observed, including decreased amplitude of evoked muscle potentials indicating loss of functioning motor units, and reduction in nerve conduction velocity, as confirmed by Russell et al (192) in the saphenous nerve. At this stage both structural and functional abnormalities can be completely reversed by the attainment of good glycaemic control with intensive insulin treatment, and partly reversed by acarbose treatment in conjunction with low dose insulin treatment. In both cases the protective effect diminished with age (193). Aldose reductase, a rate limiting enzyme in the polyol pathway associated with the conversion of glucose to sorbitol, is thought to contribute to the long-term complications of IDDM (194,195). The enzyme is located in the eye, kidney, and myelin sheath, and is activated only when hexokinase is saturated, i.e. during hyperglycaemia. The accumulation of sorbitol in these tissues and the resulting diabetic complications have been linked to depletion of *myo*-inositol content, resulting in a derangement of sodium/potassium ATPase activity. Yorek et al (196) recently demonstrated that reduced motor nerve conduction velocity and sodium/potassium ATPase activity in rats maintained on L-fucose (a potent competitive inhibitor of *myo*-inositol transport) could be reversed by *myo*-inositol supplementation, further demonstrating that myo-inositol deficiency may be a major factor in the development of neural defects associated with acute diabetic neuropathy. Administration of aldose reductase inhibitors was shown to completely prevent the characteristic nerve conduction slowing and structural abnormalities of the nodes of Ranvier, despite only partial preservation of axonal integrity. After six months of diabetes, treatment only resulted in a partial prevention of neuropathy, demonstrating the importance of additional mechanisms besides polyol pathway activation in the pathogenesis of neuropathy (197).

Without specific treatment, nerve conduction velocity falls to 85% and then 60% of normal values after 4 and 11 months of diabetes respectively. This deterioration in function is accompanied by subtle structural changes heralding the irreversible stage of neuropathy. These changes include the disappearance of paranodal axoglial junctional complexes, which anchor the terminal myelin loops to the axolemma. Loss of myelin attachment to the paranodal region leads to paranodal myelin retraction, demyelination and eventual remyelination of the nodal area in the form of " intercalated " nodes. Marked axonal atrophy and loss of myelinated fibres is observed, followed by distal Wallerian degeneration and fibre loss. Changes identical to those described in sympathetic nerves were also found in sensory ganglion cells, proximal extramedullary axons and in proximal and distal myelinated axons of the spinal dorsal columns (198). However, sequential morphometric studies have shown that the structural changes occur earlier in sensory nerves and there is a clear proximal-distal gradient of severity. After eleven months of conventionally insulin-treated diabetes, approximately 30% of animals develop vascular features with endoneurial infarcation and focal loss of myelinated fibres. Endothelial cell degeneration and capillary occlusion by platelets and fibrin are prominent features. Thus, a good correlation has been demonstrated between metabolic, morphological, and functional abnormalities but the mechanisms responsible for these changes are still unclear.

The long-term diabetic rat also shows clinical signs of autonomic neuropathy including recurrent diarrhoea, colonic dilatation, bladder dysfunction with urinary retention, and male infertility. Impaired autonomic function has been shown to fall to 72% and 51% of normal values after two and six months of diabetes respectively. Structural changes only occur after eight months and consist primarily of axonal atrophy of both myelinated and unmyelinated nerves and loss of synaptic contacts.

Thus, the hallmark of both somatic and autonomic neuropathy, is extensive axonal degeneration and this structural pathology is preceded by functional abnormalities which are reversible with treatment in the earlier stages of diabetes.

1.3.1.2.2.2. . Retinopathy

Little information is available on the effect of diabetes on the eye in the diabetic BB rat. The classical clinical features of retinopathy are not seen on ophthalmoscopy in this animal but the retinopathology has been described (199,200).

One of the earliest signs of diabetic retinopathy in the BB rat is the selective loss of pericytes and a degeneration of endothelial cells from retinal capillaries. Capillary occlusions by fibrin and platelet thrombi were also frequently observed. Light and ultrastructural morphometric techniques have demonstrated the characteristic capillary basement thickening in both the superficial and deep capillary beds of the retina. Functional damage was suggested by the demonstration of increased permeability across the retinal pigment epithelium (201).

A chronic high tissue concentration of glucose is thought to be the underlying factor that triggers retinopathy. Indeed, basement membrane thickening of retinal capillaries can be completely prevented by vigorous blood glucose control, as achieved by intensive insulin therapy (201). Again, acarbose, which led to a substantial reduction in postprandial hyperglycaemia, gave similar results. Hyperglycaemia also leads to increased metabolism of glucose through the polyol pathway which is thought to play a role in the pathogenesis of diabetic retinopathy (194,195). Immunohistochemistry studies have indeed revealed an increase in the aldose reductase content of retinal pigment epithelium and in the pericytes and endothelial cells of retinal capillaries in the

diabetic BB rat (202). Treatment with an aldose reductase inhibitor only achieved a complete prevention of basement membrane thickening in the deep, but not superficial, capillary bed of the diabetic retina (201).

Many other factors, including formation of advanced glycosylation end products are considered to have important roles in the development of diabetic retinopathy.

1.3.1.2.2.3. Nephropathy

The use of STZ- and alloxan-induced diabetic animal models in the study of nephropathy is limited due to the significant nephrotoxic effects of both drugs. Changes observed in renal function and morphology cannot be confidently attributed to the effects of diabetes alone. Despite this, there are few reports concerning the kidney in the diabetic BB rat (203-205). As was found with retinopathy, the pathological and clinical features of severe nephropathy found in human IDDM are not observed in the BB rat. Within a few months proteinuria, though not albuminuria, and glomerular basement membrane thickening were evident, and there was an increase in renal blood flow and glomerular filtration rate. Urinary protein excretion correlated significantly with the glomerular basement membrane width in diabetic rats indicating that poor glycaemic control contributed to both clinical features. Indeed, treatment with insulin decreased proteinuria and slowed the progression of glomerular basement membrane thickening. No glomerulopathy was observed, even in established diabetic animals which may be related to the lesser degree of intraglomerular hyperfusion, hyperfiltration, hypertension and PGE_2 synthesis observed in the BB rat (206). This finding was contradicted by Woehrle et al (207), who more recently reported several clearly detectable glomerular morphological changes in the diabetic BB rat, including enlargement of mesangial

space, mesangial cell proliferation, increased expression of glomerular IgG and C3 deposits, and occurence of Armanni cells. The differences observed may be due to the different insulin regimes used in these studies. Woehrle gave irregular insulin injections to his BB rats which led to a more severe form of diabetes, whereas insulin was given daily in the earlier study.

The main disadvantage of the BB rat as an animal model of diabetes relates to its increased susceptibility to pulmonary infections : sterile granulomas in lymph nodes, kidney and pancreas, and prostatic atrophy (189). The most significant lesions involve the lymph nodes, and in young BB rats, lymph nodes show variable degrees of paracortical and medullary replacement by plasmacytoid lymphocytes (208). These findings may be related to the lymphopenia commonly, but not invariably, seen in these animals. Initially it was proposed that lymphopenia was mandatory for the development of IDDM (209,210), and as this is not a feature of human IDDM, seemed to represent a major difference between IDDM in man and the BB rat. However, it is now known that not only is lymphopenia frequently present without diabetes, but also that IDDM can develop in the absence of lymphopenia (211,212). In addition, difficulty in animal care and breeding have been reported.

1.3.1.2.3. Pancreatic insulitis

Intense mononuclear cell infiltration within and around the pancreatic islets of Langerhans, i.e. insulitis, is the characteristic histopathological lesion observed at the onset of spontaneous diabetes in DP-BB rats (168). The degree of insulitis varies widely between animals and corresponds to the severity of their diabetic symptoms. Thus, mild patchy insulitis commonly exists without any detectable impairment of either glucose tolerance or insulin secretion, whilst more severe and extensive insulitis

is associated with impaired glucose tolerance and reduced glucose-induced, but not arginine-induced, insulin secretion (213). The first phase of insulin secretion is particularly affected (214). At diabetes onset there is an absolute deficiency in circulating insulin, massive insulitis and a decrease in the number and size of pancreatic islets observed (215). However, progression through these phases is not inevitable (216), and whilst IDDM never occurs without insulitis, animals with insulitis do not necessarily go on to develop IDDM.

Many methods have been developed to isolate and characterise islet inflammatory cells from diabetic and prediabetic BB rats *in situ*. These include use of serial pancreatic biopsy (217), semiquantitative methods such as immunohistochemistry in conjunction with moAb, and more recently, specific combinations of double moAb staining for candidate effector cells (218). The semiquantitative approaches do not permit concomitant population-based *in situ* islet mononuclear cell functional studies, and are difficult to interpret quantitatively because of cross-reactivity of moAb to multiple mononuclear subsets. Double staining antibody avoids such cross-reactivity.

Despite the different methodologies used, most studies find that the *in situ* infiltration of specific cell types is time dependent (219-221). Lesions are observed as early as 2-3 weeks before overt diabetes onset and are initially focal, with most islets in one pancreatic lobule showing some degree of peri-islet infiltration, whereas islets in adjacent lobules might be unaffected, i.e. islets behave as independent organs (222). Progressive spreading of the insulitis process ultimately leads to the complete destruction of islet β-cells (217,222,223) and the onset of overt diabetes in the DP-BB rat. In chronically diabetic animals, only end stage islets remain with few or no inflammatory cells present. In such animals, islet α-, δ- and pancreatic polypeptide

cells appear to be well preserved, highlighting the striking β-cell specificity of the destructive process within infiltrated islets (217,224).

1.3.1.2.4. T cell and non-T cell involvement

Both T and non-T cell mechanisms are known to be involved in the destruction of pancreatic β-cells in the DP-BB rat, and subpopulations of lymphoid cells present at different stages of insulitis have been extensively characterised.

Marked recruitment of $ED1^+$ macrophages from the circulation and their subsequent accumulation at periductal and perivascular locations adjacent to non-infiltrated islets is the earliest, possibly the first, abnormal cellular event observed (220,221). Approximately 10-14 days before the onset of diabetes, these activated cells, which are quite distinct from the resident $ED2^+$ tissue macrophages, further infiltrate adjacent islets. Substantial numbers of T lymphocytes, including helper/inducer ($CD4^+$) and cytotoxic/suppressor ($CD8^+$) T cells, and NK cells are also observed. B lymphocytes are found to a lesser degree (219,225-227).

Semiquantitative analyses suggest that just prior to, or after, the onset of diabetes, the relative percentages of *in situ* mononuclear cells follows the hierarchy of dendritic cells and macrophages, $CD4^+$ T cells, similar or lesser numbers of $CD8^+$ T cells and finally NK cells (219,221,227-229). Some infiltrating T cells show class II expression indicating that they are active (218-221,228,229). Furthermore, Signore et al (230) injected 123I-labelled interleukin-2 (IL-2) into DP-BB rats to show it specifically bound to IL-2R-bearing lymphocytes infiltrating the pancreatic islets, again indicative of a state of activation.

Recently, more quantitative and specific methods have been used to structurally analyse islet infiltrating mononuclear cells, thereby allowing comparisons to be made to numbers observed in other lymphoid organs such as spleen and blood (218,231). In the lymphopenic DP-BB rat spleen, both before and after diabetes onset, the following hierarchy of cell composition exists : B lymphocytes (40%), macrophages (30%), T cells (20%) and NK cells (10%). This composition for all cell types, except B lymphocytes, is strikingly different from normal Wistar Furth (WF) or non-lymphopenic DR-BB rats. Furthermore, analyses of splenic T cell subsets indicate that percentages of double-negative ($CD8^-CD4^-$) and activated T cells represent major cell populations. However, the *in situ* islet infiltrate mononuclear composition is distinctly different and should be more directly related to diabetes onset. NK cells were the major cell population (70%) of immune cells present during prediabetes. At diabetes onset the NK population remained high (47%), but an increased population of T cells (40%) was observed. $CD4^+$ T cells were the predominant subset (50-55% of total T cells) before and after disease onset, although double-negative $CD4^-CD8^-$ T cell (25-30%) and $CD8^+$ T cells (15-20%) were also present. Only a minority of activated T cells were reported (<3%). The role of double-negative T cells is unclear, and has been described as providing B cell help in generating pathogenic glomerular basement membrane antibodies in murine and human lupus (232,233).

Although macrophages are amongst the first immune cells to infiltrate islets, it has not been proven that they are the first cells to mediate β-cell damage. Nagy et al (234) determined the macrophage-mediated cytotoxicity in DP-BB rats of different ages compared with age-matched DR-BB controls. No enhanced islet cell killing was observed until onset of IDDM, when macrophage-mediated killing was increased in all diabetic animals. Disease onset was accompanied by insulitis (82%) and

increased cytotoxicity (55%) which is similar to the number of DP-BB rats that progressed to diabetes. These studies indicate that macrophages are involved in cell mediated islet destruction and may indeed be the first cellular effectors to result in islet killing.

Several studies (231,235) have found significantly higher percentages of splenic and peripheral blood cell macrophages in the DP-BB rat compared with those in the DR-BB and normal Wistar rat. The quantity of MHC class II molecules on circulating macrophages was shown to be unrelated to the pathogenesis of IDDM in this animal.

Alternatively, the appearance of dendritic cells has been proposed as the earliest infiltrative event in the development of diabetes (229). These cells, like macrophages, are APC, and are capable of initiating T cell mediated immune responses (236,237), suggesting an association with autoimmunity in the BB rat. Tarufi et al (238) demonstrated that splenic dendritic cells isolated from DP-BB, DR-BB and WF rats were morphologically and phenotypically indistinguishable. However, quantitative functional assays revealed that BB rat dendritic cells were more active than those isolated from WF rats in stimulating *in vitro* T cell proliferative responses. Dendritic cells also show a higher stimulatory activity upon interaction with macrophages and their derived factors, i.e. cytokines.

The presence of NK cells as the major mononuclear cell population, both before and at onset of diabetes, implicates their involvement in the pathogenesis of diabetes in the BB rat. Additionally, Like et al (239) demonstrated that *in vivo* injection of anti-CD8 moAb prevents diabetes in DP-BB rats, and since NK cells account for the majority of peripheral blood lymphocytes expressing the CD8 antigen in the DP-BB

rat, their involvement in IDDM is further implicated (239-241). However, treatment also depleted cytotoxic T lymphocytes which also express the CD8 marker. The direct role of NK cells is suggested by prevention of autoimmune diabetes in BB rats directly treated with the antiserum anti-asialo GM1 which is cytolytic (although not specifically) to NK cells (242,243). In contrast, depletion of NK cells by injection of the NK-specific moAb, 3.2.3, decreased intra-islet accumulation without preventing or delaying diabetes onset. This result suggests that NK cells are unnecessary for autoimmune islet destruction in the spontaneously diabetic BB rat and supports the role of cytotoxic T lymphocytes.

The proportion of cytotoxic $CD8^+$ T cells in the lymphopenic DP-BB rat is markedly reduced compared with non-lymphopenic controls, and the few remaining $CD8^+$ cells are widely thought to be NK cells. Bellgrau et al (244) reported that approximately 30% of thoracic duct $CD8^+$ lymphocytes express T cell receptors for antigen, thus identifying these cells as part of the T cell lineage. However, the expression of cell surface CD8 is greatly reduced, possibly explaining their consistent failure to lyse target cells susceptible to T cell mediated cytotoxicity. In contrast, normal cytotoxic activity of these cells could be demonstrated against target cell lines expressing higher than normal levels of class I MHC antigen.

Activated $CD4^+8^-$ and $CD4^-8^+$ T cells were isolated from diabetic DP-BB rats and injected into young (30 day old) large granular lymphocyte (LGL)/NK cell depleted DP-BB rats. The incidence of diabetes was not significantly altered compared with untreated recipients (40% vs 57% respectively). Thus the diabetic syndrome can be adoptively transferred in the absence of LGL/NK cells, suggesting that $CD8^+$ T cells are involved in the diabetogenic process in the BB rat. The incidence of IDDM was significantly increased when $CD4^+8^-$ and $CD4^-8^+$ T cells from diabetic DP-BB

donors were injected into LGL/NK cell depleted DP-BB recipients, compared with recipients depleted of $CD4^{-}8^{+}$ T cells or treated with an anti-CD8 moAb ($p<0.05$). These studies demonstrated that donor $CD4^{-}8^{+}$ T cells are required for adoptive transfer of the BB rat diabetic syndrome.

Rabinovitch et al (245) reported upon the ability of prediabetic and newly diabetic DP-BB rat spleen lymphoid cells to kill DR-BB, WF (MHC-compatible) and Lewis (MHC-incompatible) rat islet β-cells. Both the proportion and the islet-directed cytotoxicity of NK cells was found to be significantly greater in prediabetic and diabetic DP-BB rats than in non-diabetic DR-BB rats. The cytotoxic effect was also shown to be β-cell specific but not MHC-restricted. More recently functional studies of a quantitative nature confirmed these findings (226).

Interestingly, 80-95% of DP-BB animals that fail to develop diabetes also show inflammation of pancreatic islets, confirming the earlier observation that insulitis can be present without the subsequent onset of diabetes. Qualatitively, there was no difference between long-term normoglycaemic and prediabetic BB rats with respect to the phenotypes of infiltrating cells, but quantitatively, an enhanced amount of activated T cells expressing class II antigens and IL-2R were observed in prediabetic animals (226).

1.3.1.2.5. Involvement of MHC class II expression

The involvement of class II gene products in determining susceptibility to other autoimmune diseases has been determined, as evidenced in studies showing disease prevention using class II-specific moAb (246). Studies of the thymus in both DP-BB and DR-BB rats revealed defective expression of MHC class II on cortical epithelial

cells (247). This lesion may lead to a failure of the normal process by which autoreactive T cells are clonally deleted during ontogeny (248). Class II molecules aberrantly expressed on β-cells that become targets of autoimmunity (249) may additionally present both foreign (250) and self antigens (251). Inappropriate expression of these class II molecules could theoretically lead to the immunogenic presentation of autoantigens to helper T cells by β-cells. Indeed, transgenic mice that constitutively express class II molecules specifically on the surface of their β-cells develop islet atrophy and hyperglycaemia, but without insulitis (252,253).

However, hyperexpression of MHC class II antigens on islet β-cells has not been reported by all groups (254,255). Preliminary studies using the BB/E rat only showed occassional expression of class II molecules on β-cells 2-3 weeks prior to IDDM onset, although this phenomenon was not observed in more detailed pancreatic biopsy studies (249). At onset, expression of MHC class II molecules is occasionally observed in association with the few remaining insulin-secreting β-cells. It has been proposed that this phenomenon represents the phagocytic uptake of fragments of damaged insulin containing islets by cells such as macrophages, which express class II antigens. Seemayer et al (225) used ultrastructural studies to show the presence of macrophages containing ingested β-cell debris within the islets of diabetic BB rats. Hyperexpression of class I antigens on islets and exocrine cells has also been observed prior to the onset of diabetes in the BB rat and possibly before cellular infiltration of the pancreas (227,256,257).

1.3.1.2.6. Adoptive transfer studies

T cells are involved in the pathogenesis of the autoimmune destruction of pancreatic cells in DP-BB rats. Neonatal thymectomy prevents development of spontaneous

diabetes (258) and peripheral depletion of T cells with anti-CD5 moAb also protects weaning animals from diabetes (238,259). Other evidence for the involvement of T cells in IDDM comes from studies examining the adoptive transfer of diabetes to normal recipients.

Diabetes can also be induced in normal recipients using serum or lymphoid cells from a newly diagnosed diabetic BB rat. However, early attempts to transfer diabetes gave conflicting results. Nakhooda et al (260) reported that lymphocytes from newly detected diabetic BB rats caused the appearance of insulitis in 37% of athymic recipients. The incidence was increased to 58% if several lymphocyte injections were given, although no hyperglycaemia was reported, probably due to the small number of islets affected by insulitis (13% and 17% respectively).

Rossini (261) failed to transfer insulitis, hyperglycaemia or glucose intolerance to athymic mice and rats after injection of acutely diabetic BB rat lymphocytes, despite using a wide variety of dose regimens and pretreatments.

Koevary et al (262,263) found that pretreatment of spleen cells isolated from diabetic BB rats with the T cell mitogen concanavalin A (Con A) produced severe diabetes, insulitis and hyperglycaemia within two weeks of transfer to young (30-40 day old) DP-BB recipients, i.e. before the age at which spontaneous diabetes appears. Lymph node or spleen cells from acutely diabetic donors that had not been preincubated with Con A failed to transfer diabetes, as did administration of Con A activated lymphocytes to DR-BB or MHC-incompatible WF rats. Transfer of diabetes to these rats was only possible if recipients were immunosuppressed with a single injection of cyclophosphamide (a compound which selectively depresses cytotoxic/suppressor T cells), 24-48 hours before administration of Con A stimulated

spleen cells from acutely diabetic BB rats. However, the incidence of disease was low (15%), although no control animals became diabetic. Interestingly, prophylactic insulin treatment has also been reported to prevent the transfer of diabetes by Con A activated diabetic BB spleen cells in the recipient BB rat and completely prevent the incidence of diabetes (264).

Like et al (265) also reported that immunosuppression of DR-BB recipients with cyclophosphamide or anti-lymphocyte serum (ALS), or neonatal thymectomy was necessary for adoptive transfer of diabetes. This same adoptive transfer procedure could also induce disease in F_1 hybrid offspring of BB x Lewis ($RT1^l$), BB x Brown Norway ($RT1^n$), BB x Yashida ($RT1^u$) and BB x NEDH ($RT1^g$) matings.

The possible role of soluble factors in the transfer of diabetes has also been examined by injecting young (30-35 day old) DP-BB rats with culture supernatants from Con A-treated spleen cells of acutely diabetic BB rats. Diabetes developed in 25% of recipients (266). The nature of the diabetogenic factor is uncertain but cytokines such as IL-1, TNF-α, and IFN-γ are likely candidates (245).

It has been shown that Con A activation of T lymphocytes requires the presence of macrophages (267). Other mitogens, namely phorbol myrisate acetate (PMA; a direct activator of protein kinase C) and ionomycin (I; a Ca^{2+} ionophore) can reportedly activate lymphocytes in the absence of accessory cells (268), and when used in conjunction were shown to improve *in vitro* proliferation of diabetic BB splenocyte subsets in comparison to Con A.

The incidence of adoptive transfer was similar when DP-BB splenocytes were injected into 30 day old DP-BB rats after activation with either PMA + I + IL-2 or Con A

(57% vs 50% respectively). However, injection of a purified splenocyte subset containing T lymphocytes and LGL, but depleted of macrophages, isolated from DP-BB animals after PMA + I + IL-2 activation resulted in a significantly higher incidence of adoptive transfer (90%, $p<0.05$), even when cells were injected in small numbers. Such autoreactive cells were found in the young DP-BB rat and further studies determined that preactivated and enriched $CD4^+$ T lymphocytes, but not B lymphocytes, induced the adoptive transfer of diabetes in recipients, demonstrating that β-cell destruction can be induced by this T cell subset (269).

IDDM can be induced in sublethally irradiated NOD mice receiving high doses of cyclophosphamide after transfer of diabetic spleen cells. Short-term treatment with a non-depleting CD4 moAb prevented transfer of diabetes (270) and cyclophosphamide-induced diabetes (262) long after cessation of moAb treatment. However, cyclophosphamide can abrogate this induced tolerance without the requirement for newly generated T cells by the thymus.

Several groups have shown that the adoptive transfer of diabetes can be prevented. Barlow and Like (271) reported anti-CD2 moAb treatment of the DP-BB recipient prevented the transfer of diabetes with Con A-treated splenocytes from diabetic BB donors. This treatment depleted the recipients of $CD4^+$ T lymphocytes without the depletion of NK cells or $CD8^+$ T lymphocytes providing further evidence for $CD4^+$ T lymphocyte involvement in the transfer of diabetes.

Adoptive transfer is known to be facilitated by prior *in vivo* depletion of $RT6^+$ T lymphocytes using an RT6-specific cytotoxic moAb. Diabetes can be induced in approximately 50% of DR-BB rats treated from 30 days of age (273), and RT6-depleted DR-BB splenocytes can transfer diabetes to athymic nude rats with no

prior *in vitro* mitogen activation (274), strongly suggesting that both DP-BB and DR-BB rats circulate autoreactive cells that have escaped clonal deletion. Conversely, transfusion of $RT6^+$ T lymphocytes from DR-BB to DP-BB rats prevents diabetes. It has also been reported that a single transfusion of lymphocytes from long-term normoglycaemic DP-BB rats into prediabetic DP-BB recipients decreased the incidence of diabetes (275). These results taken together provide evidence for suppression of autoreactive T cells in BB rat diabetes, and suggest two defects in these animals : one causing autoreactive cells to appear in the peripheral tissues, and another at the level of stem cells causing lymphopenia (276,277). Depletion of $RT6^+$ T cells in the DR-BB in essence makes these animals the functional equivalent of the DP-BB rat.

1.3.1.3. IMMUNOPATHOGENESIS

1.3.1.3.1. Humoral immunity

The identity of the putative β-cell antigen that is the target for autoimmune attack has still to be determined. The presence of abnormal surface antigen(s) in the diabetic pancreas, either congenital or induced e.g. by viral infection, has been suggested but no evidence to support this theory has been reported.

As in human IDDM, indirect evidence that the humoral arm of the immune system contributes to the pathogenesis of autoimmune diabetes in the BB rat is suggested by the appearance of autoantibodies reacting against various tissues and proteins, both in the prediabetic stage and at diabetes onset. These include antibodies to gastric parietal, smooth muscle and thyroid cells and lymphocytes (162,278-280). ICSA have been detected 4-8 weeks before diabetes onset (281-283) and IAA have been

reported (256,282,284). A 64 kD protein, now recogmised as GAD, has been proposed as a potential autoantigen in humans and animal models of diabetes (285). However, the 64 kD protein present in the BB rat remains to be proven to be GAD (162). Antibodies to pituitary, adrenal, testis and ovary cells and islet cell cytoplasmic components (278,279) have not been reported in the BB rat and no mononuclear cell infiltration is observed in these non-pancreatic tissues.

ICSA were first reported in approximately 85% of newly diagnosed diabetic BB rats by Dyrberg et al (286) using an ^{125}I-protein A radioligand assay with dispersed Wistar rat islet cells. Subsequently, using the rat insulinoma cell line RINm5F (287), ICSA were also shown to be present in the prediabetic period and in some animals at weaning, long before the presence of any morphological and metabolic disturbances associated with IDDM. However, the use of this autoantibody as a predictive marker for the onset of diabetes is limited because of conflicting findings between groups. Pollard et al (288) detected ICSA in 100% of prediabetic and diabetic animals using an indirect immunofluorescence assay on pancreatic islet cell suspensions, whereas Pipeleers et al (281) only reported ICSA in the diabetic, but not the prediabetic, rat. The majority of ICSA are capable of complement-mediated cytolytic activity against pancreatic islets *in vitro* (289,290), but no direct pathogenic role of these autoantibodies has been demonstrated *in vivo*. Thus, ICSA may represent a secondary phenomenon resulting from the destruction of islet cells.

The presence of IAA has been reported in the BB rat but the finding is disputed. Diaz et al (291) followed the time course of IAA in the DP-BB rat by ELISA using purified rat insulin, and found increased IAA frequency and titre with time. No DP-BB rats were IAA positive at 45 days of age, whereas 89% were positive at day 90 and 100% were positive thereafter. This pattern was similar for the DR-BB rat

with 100% IAA positive at day 90. Wistar controls were IAA negative at 105 days, with only an occassional positive sera at 120 days of age. IAA therefore appeared early in the majority of both DP-BB and DR-BB rats and much later, if at all, in controls, suggesting that IAA is a strain-related phenomenon rather than a marker for either insulitis or the development of IDDM in the BB rat. Markholst et al (292) attempted to resolve this controversy by measuring IAA in well-characterised inbred BB rats using a radioligand assay with ^{125}I-labelled rat insulin and an ELISA method using rat insulin as antigen. DP-BB rats were studied from 15 weeks of age to the last week before onset, and at onset of diabetes. Age-matched non-diabetic DP-BB and DR-BB animals were used as controls. Levels of antibody binding increased slightly with increasing age in all groups. In contrast to previous studies, a low frequency of IAA was only detected at diabetes onset and was undetectable in the prediabteic period. Markholst et al proposed that differences in analytical method, animal age and inappropriate comparison of sera from BB and Wistar rats may account for the variable results from different studies.

Dean et al (282) determined the presence of ICSA and IAA in DP- and DR-BB/E rats. A higher prevalence for ICSA than IAA was reported and a higher incidence of both autoantibodies was demonstrated in DP-BB than in DR-BB rats. There was however no correlation between ICSA and IAA in individual animals and whereas a highly significant association was observed between ICSA and the development of diabetes ($p<0.005$), IAA was only weakly associated with diabetes ($p<0.03$). There was no significant association of IAA with DP- in comparison to DR-BB rats, although positive IAA values were significantly elevated in the former compared with the latter ($p<0.01$).

The 64 kD islet cell protein is expressed at high levels in brain, and at lower levels in islets, kidney, liver, testis, ovary and pituitary, thyroid and adrenal glands in the diabetic BB rat (293). 64 kD protein expression in rat pancreatic islets was recently shown to increase upon high glucose stimulation, mediated via an increase of 64 kD protein mRNA expression (294). The rate of 64 kD protein expression therefore followed the pattern of insulin release suggesting that the regulation of 64 kD protein expression in the β-cell co-varies with the expression of the insulin gene.

Rat islets have recently been shown to contain autoantibodies to 64 and 67 kD proteins which are encoded by different genes in the rat (295), and are β-cell specific in their expression. Autoantibodies against the 64 kD were initially detected in rat islet cell fractions using an immunoprecipitation technique (296). All diabetic and 92% of prediabetic DP-BB rats were positive for 64 kD protein antibodies. However, a lower frequency was observed in the DR-BB rats, and WF rats were negative for 64 kD protein antibodies. More recently, ELISA using GAD-enriched rat brain extract has been used for the detection of this autoantibody in the BB/OK rat, and similar results have been reported (297). Sera of non-diabetic and diabetic DP-BB rats showed a 97% and 88% antibody prevalence respectively at 120 days of age compared with none of the age-matched Lewis control group. Antibodies to 64 kD protein could be detected approximately 50 days before diabetic manifestation in both diabetic (84.6%) and non-diabetic (71.4%) DP-BB groups, indicating a close association of autoantibody with genetic susceptibility to diabetes. However it is not of predictive value with respect to the onset of diabetes in BB/OK rats.

Autoantibody to a rat islet cell-protein of 38 kD has been detected in sera of 30-day-old DP-BB rats using immunoprecipitation and Western blotting methods (298). Over 90% of DP-BB rats in which the antibody was detected eventually

developed IDDM and the antibody disappeared within two weeks of onset. The anti-38 kD islet cell autoantibody was not observed in the sera of 5-20 day old DP-BB or WF rats, and appeared to be β-cell dependent, since the autoantibody disappeared after almost complete depletion of β-cells, and was consistently present as long as β-cells remained. Identification of the autoantibody will be invaluable for study of the molecular nature of target islet cell autoantigen(s) associated with the induction of autoimmunity in the DP-BB rat.

Recently, a new quantitative method to measure autoantibodies directed against T lymphocytes based on two-colour fluorescence labelling of mononuclear cells and two-colour flow cytometry has been developed (299). As mentioned earlier, lymphocyte antibodies are present before insulitis and diabetes onset in the BB rat, and show a positive correlation with ICSA. Lymphocyte autoantibodies bound both $CD4^+$ and $CD8^+$ T cells and were present in 90% of BB rats at diabetes onset compared with only 11% of Wistar rats. At 75 days of age, 83% of BB rats which later became diabetic were positive for lymphocyte antibodies, compared with 15% of their littermates maintaining normal glucose tolerance. Using this novel method, the presence of lymphocyte autoantibodies could predict the development of diabetes with a sensitivity of 83% and a specificity of 85% in BB rats.

1.3.1.3.2. Cellular immunity

The severe lymphopenia associated with the diabetic state in the BB rat is well established (299,300) and is present in affected animals from the preweaning period (301). Until recently, it was thought that lymphopenia was obligatory for the development of IDDM (209,210). However, it is now clear that lymphopenia is frequently present without diabetes, and conversely IDDM can develop in the absence

of the lymphopenic trait, although not necessarily of a high frequency (211,212). Indeed a newly derived diabetes-susceptible rat strain, designated Long Evans Tokushima Lean and developed from the $RT1^u$-bearing Long Evans stock, appears not to be lymphopenic (302). However the development of diabetes is greatly favoured by the presence of lymphopenia (210,303).

The lymphopenia affects both the T and B lymphocyte subsets and both populations are depressed, with T cells being affected to a greater degree. The proportion of B cells are reported to be normal (300) or slightly increased (304) but in terms of absolute numbers a decrease in the population is apparent (305).

The T cell lymphopenia is largely accounted for by a total or very near total absence of mature $CD5^+$ T cells in peripheral blood, spleen and lymph nodes expressing the T cell differentiation alloantigen RT6 (306,307). Concomitantly, there is also a markedly reduced level of $CD4^+$ helper/inducer T cells and a total (209,308) or near total absence (309) of the $CD8^+$ cytotoxic/suppressor T cell subset. A greatly diminished density of CD8 antigen on the few remaining $CD8^+$ cells is observed.

The gene encoding RT6 is on chromosome 1 in the rat (310) and its RT6 allogeneic system comprises of at least two alleles, a and b. The RT6a gene encodes the RT6.1 alloantigen and RT6b encodes RT6.2. Biochemical analysis of the RT6.2 antigen from normal rats indicates that it is a polypeptide anchored to the cell membrane through a phosphatidyl inositol linkage (311). Evidence of an intact phosphatidyl inositol linkage of the RT6 molecule was demonstrated by immunoprecipitation of the phosphatidyl inositol protein (306) or treatment with a phosphatidyl inositol specific phospholipase C (311). Both treatments led to a markedly reduced RT6 density on T cells indicating that the RT6.1 antigen is correctly processed and folded in diabetic

BB rat lymph node cells. This data demonstrates that the near total absence of $RT6^+$ T cells in the BB rat is unlikely to be caused by a defect in the RT6 gene expression per se. Defects in gene regulation or other cellular defects leading to premature cell death in the T cell lineage alone or in combination may instead be responsible.

The RT6 alloantigen is expressed on approximately 60% of the peripheral T cells in the normal or DR-BB rat, including approximately 50% of $CD4^+$ and 70-80% of $CD8^+$ T cells (306). RT6 is a maturational antigen found on B lymphocytes, brain, thymocytes or bone marrow cells, but not on neonatal peripheral T cells, and adult levels of expression are not achieved until approximately 60 days of age (312). Bone marrow adoptive transfer studies using DP-BB and WF rats have traced the inability of DP-BB rats to generate $RT6^+$ cells to developmental defects of their bone marrow-derived stem cells (313-315). These defects appear not to be expressed at the pre-thymic developmental stage of T cell precursors (316), therefore RT6 represents a marker for post-thymic T lymphocyte maturation.

Given the exclusive expression of RT6 antigen on mature lymphocytes, it has been suggested that the peripheral T cell pool of DP-BB rats consists almost exclusively of immature lymphocytes. This hypothesis is supported by the defective expression of the CD45R marker on DP-BB rat T lymphocytes (317). CD45R is a surface antigen found on both T and B lymphocytes (318) and 33% of $RT6^+$ T cells in the DR-BB rat (319), which subdivides the mature $CD4^+$ T cell subset into suppressor/inducer ($CD4^+/CD45R^+$) and memory helper/inducer ($CD4^+/CD45R^-$) populations. Interestingly, athymic rats transfused with $CD45R^+$ T cells develop autoimmune symptoms that do not occur if $CD45R^-$ cells are present (320).

T lymphocytes expressing RT6 are important regulators of the autoimmune process in the BB rat. The regulatory function of $RT6^+$ T cells was first suggested by the observation that spleen cell transfusions from normal rats prevents diabetes in the DP-BB rat if the treatment led to engraftment of the $RT6^+$ donor T cells (321,322). Conversely, treatment of non-lymphopenic DR-BB rats circulating normal RT6 cells with anti-RT6 moAb from 30 days of age led to the destruction of $RT6^+$ T cells and increased the frequency of diabetes from <1% to >50% within 2-3 weeks. None of the controls developed IDDM (273,319). If anti-RT6 moAb treatment was delayed to 60 days of age, diabetes did not occur suggesting the existence of a critical period during which the autoimmune process is initiated and afterwhich the regulatory population of $RT6^+$ cells cannot reverse.

Angelillo et al (315) used genetic complementation studies to show that the DP-BB rat T lymphocytes contain an intact RT6 gene but failed to express the RT6.1 alloantigen. Southern blot analysis of DP-BB genomic DNA showed that this gene was not grossly altered (323). Possible reasons for this lack of expression include functional absence of an accessory factor provided by $RT6^+$ T cells, lack of appropriate induction signals, or a failure to generate T cells which can be induced to express RT6 during post-thymic differentiation (324). T lymphocytes released from the DP-BB thymus suffer from a maturational block which does not allow induction of RT6. As these animals are lymphopenic, despite a normal number of T cells being released from their thymus as judged by the number of $Thy1^+$ lymph node cells (323), it may be concluded that in the DP-BB rat most thymic emigrants die before RT6 can be expressed.

Recently, several studies have questioned the reported absence of $RT6^+$ cells in the DP-BB rat. Lang and Kastern (325) reported that the RT6 subset is present in the

diabetic BB rat in normal proportions, when based upon total lymphocyte number, and suggest that the widely reported decrease in RT6 cell numbers results from, rather than causes, the lymphopenia. Kastern took advantage of the close linkage between RT6 and the albino genes on rat chromosome 1 to verify the exact role of the RT6 gene in the inheritance of lymphopenia. The F_2 generation of outcrosses between diabetic BB and Long Evans Hooded (LEH) rats required both homologues of chromosome 1 from the diabetic BB grandparent in order to be albino. Although most of the albino F_2 rats should also have been homozygous for the BB RT6 gene, no increase in the incidence of IDDM (3.7%) and lymphopenia (22.2%) was observed. Furthermore, the RT6 gene was fully functional and was expressed normally on the surface of T cells of peripheral blood lymphocytes within the F_2 population. This suggested that neither a defective RT6 gene nor an altered expression of RT6 antigen could account for diabetes or lymphopenia in the DP-BB rat.

Crisa et al (326) used molecular and biochemical procedures to investigate the absence of the $RT6^+$ T cell subset in the diabetic BB rat. A mRNA encoding RT6 protein was present in the DP-BB spleen cells, and nucleotide sequencing of this transcript revealed an intact coding sequence for the RT6.1 alloantigen. Sensitive chemiluminescent assay of lymph node cell detergent extracts showed that the RT6 mRNA was translated *in vivo*, and quantitatively these cells expressed <10% of the RT6.1 protein found on DR-BB rat lymph node cells.

Further evidence demonstrating that the DP-BB rat possesses a functional RT6 gene came from analysis of the isolated intestinal intraepithelium lymphocytes (IEL), using an immunofluorescence method (324). The normal rat is known to express a very high density of RT6 molecules on the IEL. The population of IEL in the diabetic rat

was found to be reduced by 50% and consisted predominantly of $CD4^+$ T cells, the majority of which clearly expressed RT6. However, the RT6 cell surface expression was still only approximately 10% of that observed in normal rats. These observations may be explained when considering that IEL may be a thymus independent T cell population (310). It is possible that $RT6^+$ IEL are therefore derived from a different precursor population and mature along a different developmental pathway. If RT6 expression is an early differentiation step in the maturation of IEL, then IEL of DP-BB rats may reach the stage of RT6 expression before the maturational block becomes effective. Thymus-dependent peripheral T cells of the same rat have an unusually short life span in comparison and may not live long enough to reach the stage of complete maturity characterised by surface expression of T cell differentation markers such as RT6 and CD45R (327). This theory is supported by McKeever et al (274) who reported that the level of $CD5^+$ T cells in DP-BB rat lymph nodes falls from approximately 29% to 2%, 25 days after thymectomy at 4-6 weeks of age. This data further suggests that $CD5^+$ T cells in diabetic rats have a very short lifespan, and because cells committed to the RT6 lineage do not express the RT6 antigen on their surface until 4-14 days after release into the circulation (306,327), this may be another explanation for the lack of $RT6^+$ T cells in the diabetic BB rat. Particularly relevant to this finding is the recent report of Jacob et al (328), who described the chromosomal localization of a DP-BB rat gene, designated *Lyp*, which may be responsible for lymphopenia. This gene and the RT6 gene are thought to be different (325,328). In order to ascertain whether the shortened life span of diabetic T cells is attributable to under- or overexpression of the *Lyp* gene product, further studies involving the cloning and characterization of the *Lyp* gene are necessary.

1.3.1.4. GENETICS

Inheritance of IDDM in BB rats appears to be polygenic involving at least two independent recessive genes (329). One of these genes is associated with the rat major histocompatibility complex (MHC) which is designated RT1. The RT1 complex encodes three types of class I molecules located at the RT1.A, RT1.E and RT1.C loci, and two types of class II molecules at the RT1.B and RT1.D loci. Studies have shown that expression of diabetes is independent of class I haplotype, but requires the presence of at least one class II $RT1^{u}$ allele (330,331). However, intercross studies indicate that the u allele of the BB rat is not a unique diabetogenic variant allele, since u alleles derived from normal rat strains also confer susceptibility (332,333). Diabetic male BB rats crossed with inbred female Lewis ($RT1^{l}$) or Buffalo ($RT1^{b}$) rat strains demonstrated that all diabetic animals were either homozygous ($RT1^{u/u}$) or heterozygous ($RT1^{u/l}$ or $RT1^{u/b}$) for the u haplotype. There was no apparent difference between BB rats that were homozygous or heterozygous for the u haplotype in relation to age of diabetes onset or sex related incidence of diabetes, although IDDM occurs more frequently in homozygous $RT1^{u/u}$ animals (334). This observation is similar to the human situation, where only one copy of the permissive class II allele is sufficient to confer susceptibility. Thus, as in diabetic sibships where affected siblings have one, and in many cases both, haplotypes in common with the propositus irrespective of the HLA determinants that make up the haplotype, so within BB rat litters where MHC haplotypes are segregating, diabetes occurs in animals with the same genotype. If the first animal to develop diabetes is $RT1^{u/u}$ then subsequent diabetic animals will also be $RT1^{u/u}$. Conversely if the first diabetic is $RT1^{u/x}$ then subsequent litter mates are also $RT1^{u/x}$.

Several genetic studies indicate that the class II region of the BB rat RT1 complex is involved with the development of disease (332,335). Boitard et al (336) demonstrated that treatment of BB rats *in vivo* with anti-RT1.D but not RT1.B monoclonal antibody reduced the frequency of diabetes and thyroiditis, emphasising the importance of MHC class II gene products in these animals.

The possible association of variant or unique class II MHC molecules with IDDM susceptibility has been investigated by comparing the $RT1^{u}$ allele carried by BB rats with that of other strains using various biochemical techniques. These techniques show that the gene product of the BB rat $RT1^{u}$ haplotype is indistinguishable from the $RT1^{u}$ gene products of normal WF rats (337). The nucleotide sequences of class II (RT1.B and RT1.D) mRNA's from DP-BB, DR-BB and normal WF rats have also been determined (338). The first external domains of the β-chains of RT1.B and RT1.D molecules were shown to be identical for the u haplotype in the BB rats, but differed from the WF class II genes. These findings establish that the class II gene sequences of the BB rat may be unique and that these class II molecules of the u haplotype support the autoimmune response in the BB rat.

Speculation about mechanisms of class II involvement with diabetes susceptibility include the concept that abberant expression of class II genes and/or structural differences in class II molecules provide the basis for disease susceptibility by allowing the presentation of environmental antigen and/or self antigen to the immune system. This hypothesis is strengthened by the elevated steady-state mRNA levels of the RTI.D, but not RT1.B, genes in the lymphocytes of DP-BB rats alone, indicating differences in their class II regulation.

The amino acid serine at position 57 in both class II RT1.B and RT1.D β-chains of both DP-BB and DR-BB rats appears to be positively associated with IDDM, whereas the presence of aspartate at this location is neutral or negatively associated with diabetes. This phenomenon is also found in the human HLA-DQ β-chain. Interestingly, non-diabetic Lewis ($RT1^l$) and Buffalo ($RT1^b$) rats possess a serine residue rather than an aspartate residue at this position (337). This suggests that the allelic amino acid at position 57 of class II β-chain sequences does not serve as an autoimmune disease susceptibility marker in the BB rat. However, the absence of differences in the class II β-chain sequences of DP-BB and DR-BB rats does not mean that these genes are not involved in IDDM susceptibility. Other unidentified class II allelic differences or factors may be involved, and due to the polygenic nature of spontaneous diabetes in the BB rat, these genes or factors may lie outside the MHC region.

The RT1-linked diabetogenic gene of the BB rat was further defined by studying RFLP in order to determine any MHC differences between DP-BB and DR-BB rats at the genomic level. Both class I and II MHC probes were used. Using the restriction endonucleases BamH1 and EcoR1 in conjunction with an I-Aα (class II mouse MHC) gene probe, Buse et al (339) distinguished four polymorphic chromosome types (Ia, Ib, IIa and IIb) in the DR-BB population, only one of which is found in the DP-BB rat (all homozygous for the type IIa chromosome). From this finding, the RT1-linked diabetogenic gene of the BB rat is linked to a defined gene of the type IIa chromosome. The same pattern of polymorphisms, though of different molecular weights, was found using a HLA-DCβ probe (human class II light chain). In contrast, Kastern et al (340) reported no detection of polymorphisms using this probe.

Recent evidence has suggested the possibility of a uniquely important role for faulty class I expression in diabetes pathogenesis in humans and NOD mice (341). Class I molecules are important restrictive elements for T cell selection of targets and play a key role in antigen presentation. Indeed, hyperexpression of class I antigens appears to occur on islet and exocrine cells in diabetic animals, and hyperexpression on vascular endothelial cells may be the earliest abnormality in the development of diabetes in BB rats (256). Enhanced levels of MHC class I heavy-chain mRNA in pancreatic islets has also been demonstrated in DP-BB rats before overt inflammation and onset of diabetes (257). Immunohistochemistry revealed enhanced class I antigen expression in all pancreatic islets of newly diabetic animals without induction of class II.

Polymorphisms that further subdivided the DP-BB and DR-BB rat were found and characterised using a mouse class I gene as probe (340). Specifically, a 2 kB BamH1 fragment was present in all non-diabetic, but not diabetic, BB rats. Similar polymorphisms were observed with various other restriction enzymes, paticularly Xbal, Hind II and Sac I, further demonstrating that the BB rat diabetic syndrome may be linked to differences in class I MHC genes.

A gene or gene cluster determining T cell lymphopenia is also strongly associated with the development of autoimmune diabetes in DP-BB rats. Lymphopenia behaves as an autosomal recessive trait and segregates independently of the RT1 haplotype (303,342). However, lymphopenia is only permissive and not obligatory for diabetes development, as indicated by the sporadic cases of diabetes in non-lymphopenic DR-BB sublines. Furthermore, in neonatally thymectomised DP-BB rats, i.e. rats lacking T cells, diabetes did not occur (258).

The role of the thymus was therefore investigated with respect to the lymphopenic state and the pathogenesis of autoimmune diabetes (343). Neonatal BB rats were inoculated with MHC-compatible, T cell deficient WF bone marrow cells, thus rendering the host unresponsive to WF antigens. The animals were then given a thymus allograft from a normal non-lymphopenic WF donor during early adulthood. There was no prevention of diabetes and no significant alteration in the recipient lymphopenic profile, suggesting neither β-cell destruction, nor lymphopenia were consequences of a defective thymus and/or a result of impaired intrathymic maturation. A stem cell or prothymocyte defect within the central lymphoid compartment of the BB rat may explain both the lymphopenia and the defective immunoregulation that ultimately permits the expression of diabetes in the BB rat.

Guberski et al (342) and Markholst et al (303) used classical genetic studies to confirm the permissive relationship between lymphopenia and diabetes and the recessive nature of both. Furthermore, it was suggested that the combination of genes responsible for diabetes among lymphopenic and non-lymphopenic BB rats may be distinct.

In summary, genetic studies have shown that the inheritance of IDDM in the spontaneously diabetic BB rat is polygenic, and at least two genes are necessary for its development. One gene is linked to the rat MHC, specifically the class II region, and the presence of at least one u haplotype appears to be a necessary prerequisite for IDDM development. The MHC development of diabetes in the BB rat can be further mapped to the class II RT1.D locus, although there is no evidence to indicate an abnormal class II RT1.D gene product. The other implicated diabetogenic gene is autosomal recessive and determines the profound T lymphopenia observed in this

animal. Other non-MHC genes also modify disease penetrance and the time of onset of diabetes (261).

1.3.1.5. INVOLVEMENT OF CYTOKINES

As noted earlier, macrophages are amongst the first infiltrating cells in the DP-BB rat islet and may therefore be involved in islet cell injury. Administration of silica, which is selectively toxic to macrophages, prevented insulitis and diabetes in young BB rats (344-346). Furthermore, DP-BB neonates injected with Con-A-activated splenocytes from silica treated, but not untreated, diabetic rats were also protected from IDDM and insulitis. Similar protection was observed for both DP-BB and RT6-depleted DR-BB rats fed a diet deficient in essential fatty acids, which is known to affect macrophage number and function (347).

Macrophage-mediated injury could be caused by cytokine release. IL-1 is a cytokine produced in an antigen non-specific, non-MHC-restricted way, primarily by activated macrophages and monocytes, but also by several other cell types (348). Novel studies by Mandrup-Poulsen et al (349) showed that cytokines from human mononuclear cells caused both functional and structural damage to isolated rat islet β-cells. It was later demonstrated that the cytotoxic action of cytokines was exerted through IL-1 activity specifically. IL-1β is known to have complex dual effects on islet β-cell function : at low concentrations (0.5 U/ml) and/or short exposures (60-90 minutes), IL-1β stimulates insulin synthesis and secretion. Conversely, at high concentrations (5 U/ml) and/or long exposures (>24 hours), this cytokine caused an inhibition of biosynthesis and insulin secretion (350,351). The β-cell response to IL-1β was also affected by ambient glucose concentration. High IL-1β and glucose concentrations caused an earlier and more pronounced

inhibition of insulin secretion. In contrast, low IL-1β and non-stimulatory glucose concentrations delayed the shift from stimulation to inhibition. This glucose effect, which is also observed with leucine, suggests that the IL-1β effect may depend upon the degree of β-cell activity, i.e. increasing IL-1β concentration, exposure time and β-cell activity resulted in increased islet disintegration. Thus, the resting β-cell is more resistant to IL-1β-mediated impairment than the working β-cell. Jobe et al (352) confirmed these findings by demonstrating that daily low dose injections of IL-1β decreased the frequency of IDDM in the DP-BB rat. Conversely, high doses of IL-1β induced an earlier than normal onset of diabetes, and this was associated with increased lymphocyte and neutrophil numbers.

TNF-α is also secreted by activated macrophages and monocytes. Although TNF-α has virtually no effect on islet β-cell function alone, low concentrations of TNF-α potentiate the cytotoxic effect of IL-1β on pancreatic islets (353,354). In contrast to the normal IL-1 production by activated macrophages in DP-BB rats, TNF-α production is probably upregulated (355). Rothe et al (355) further reported that activated peritoneal macrophages secrete strikingly higher amounts of TNF-α than DR-BB or Wistar rat macrophages. Enhanced TNF-α was detected prior to occurrence of insulitis, and precursor cell-derived macrophages from the diabetic DP-BB rat bone marrow also showed upregulated TNF-α secretion upon challenge with IFN-γ. This reported TNF-α hypersecretion may affect thymic and post-thymic T cell maturation and promote pancreatic islet inflammation, thus contributing to the autoimmune diabetes. In contrast, prevention of diabetes and insulitis after treatment of BB rats with TNF-α suggests that deficient TNF-α production may be involved in the immunopathologies of autoimmune diabetes (356). TNF-α production by peritoneal macrophages from DP-BB rats was significantly decreased, both in the basal state and after stimulation, with either IFN-γ or lipopolysaccharide *in vivo* and

in vitro, as compared with control rats. Deficient macrophage production of TNF-α may therefore be casually related to development of IDDM in the DP-BB rat (357).

The mechanism of this cytokine-mediated β-cell cytotoxic effect is uncertain. It has been suggested that induction of intracellular free oxygen ($O2^{\bullet-}$), hydroxide ($OH^{\bullet}$), and nitric oxide ($NO^{\bullet}$) radicals may be involved (59, 358), since IL-1 has been shown to produce free radicals in other cells (359,360). NO is synthesised from L-arginine by the enzyme NO synthase (NOS). Two different types of NO synthase exist both of which depend upon the cofactor NADPH for activity. Small physiological amounts of NO are produced by the calcium-dependent isoform, whereas large cytotoxic doses are generated by the calcium-independent, cytokine-inducible isoform (361). These cytotoxic effects are thought to be caused by NO-mediated destruction of iron-sulphur centres of iron containing enzymes and result in an impairment of mitochondrial function and DNA synthesis (362). Recently, Fehsel et al (363) reported that NO-induced islet cell lysis was preceded by DNA damage. Since DNA damage is a mechanism of cytokine-induced cell death in certain cell types, Rabinovitch et al (364) measured DNA fragmentation in rat islets to determine the cytotoxic effects of cytokines at the nuclear level. Although the individual cytokines IL-1, TNF and IFN-γ inhibited insulin release from rat islets, no DNA fragmentation or islet destruction was observed unless the three cytokines were used in combination. The cytokine-induced DNA fragmentation was found to precede cell lysis in islet β-cell lines. These findings identify DNA as an early target of cytokine action in islet β-cells and implicates DNA fragmentation as a mechanism of cytokine-induced β-cell destruction. Administration of the free-radical scavenger nicotinamide prevented both DNA fragmentation and IL-1-mediated β-cell damage in isolated rat islets. Nicotinamide also prevented islet graft destruction in the NOD

mouse (365). In addition, antioxidants have been shown to prevent (366) or reduce the incidence of (367) diabetes in NOD mice.

1.3.1.6. THE β-CELL AND IDDM

Since normal rat islets transplanted into DP-BB rats are destroyed by recurrent autoimmunity, islets from diabetic BB rats do not appear to be antigenically unique (368,369). As discussed earlier, the functional state of the β-cell may determine its susceptibility to autoimmune destruction. Indeed, chronic insulin administration to young DP-BB rats significantly decreased the incidence of diabetes and insulitis (370,371). Furthermore, Gotfredsen et al (371) noted that despite the discontinuation of insulin administration at 140 days of age, the majority of treated DP-BB rats remained normoglycaemic until 230 days of age. Diazoxide, an insulin secretion inhibitor, also decreased the incidence of overt diabetes by approximately 50%, demonstrating that the protective effect of insulin was not simply a hypoglycaemic effect (372). However, the mechanisms by which a decrease in the insulin secretory activity protects against the autoimmune process has yet to be determined.

Other studies have demonstrated a selective survival of neonatal pancreatic islets as compared with adult islets transplanted in acutely diabetic DP-BB rats (373), suggesting that the putative β-cell target antigen might be absent in neonatal BB rat β-cells. Additionally, neonatal metabolic stimulation of β-cells has been reported to prevent the development of diabetes in BB rats (374). Taken together, these studies may suggest that an aberration in the temporal expression of potential β-cell autoantigens early in life, at a time when tolerance to self antigens is

established, may lead to islet autoimmunity. Expression of this putative autoantigen appears to be related to the functional maturity of the β-cells.

In summary, *in vitro* studies have demonstrated the cytotoxicity of the cytokines IL-1β, TNF-α and IFN-γ either alone or in combination, to the β-cell, possibly involving the generation of free radicals. These cytokines are synthesised by activated macrophages which are present in the initial pancreatic islet infiltrate in the pre-diabetic BB rat. The factor initiating the insulitis and subsequent autoimmune destruction of the islet β-cell is unknown. Cytokines, if present at high local concentrations in the islet, may cause β-cell cytolysis, resulting in the release of sequestered antigen(s) from the cell. Macrophages and dendritic cells bearing MHC class II molecules can act as antigen presenting cells and may initiate autoimmune processes by presenting the putative β-cell antigen(s) to autoreactive helper T cells also present in the insulitis. This recognition results in the activation of helper T cells, involving both the growth of antigen-specific T lymphocyte clones and their differentiation to an effector function, which may involve secretion of bioactive substances or the development of a specific capacity such as cytotoxicity or phagocytosis. The damaged β-cells hyperexpress class I molecules and thereby elicit an increased attack by cytotoxic T cells. Activated macrophages in the infiltrate produce free radicals and β-cells are further damaged. Proteins normally sequestered within the β-cell are released, and as they are unfamiliar to the immune system, they are perceived as foreign and autoantibodies are produced against them. This scenario may represent the sequence of events leading to the autoimmune destruction of islet β-cells. As more and more insulin-producing cells are destroyed, the remaining healthy β-cells become more overworked. This hyperactivity stresses the cells and perhaps leads to an increased susceptibility to autoimmune attack. Once an

insufficient number of β-cells remain, the clinical expression of IDDM in the BB rat is observed.

1.3.1.7. ENVIRONMENTAL FACTORS

One of the many unsolved problems associated with the inheritance of human IDDM is the observation that approximately 50% of all pairs of monozygotic twins are discordant for diabetes (375). A similar situation pertains to the BB rat where even in long established inbred colonies the frequency of diabetes is still only 50-80%. This suggests that environmental factors may play a possible role in the expression and incidence of diabetes.

1.3.1.7.1. Viral infection

An early study (376) into the influence of infectious agents on the incidence of diabetes in the spontaneously diabetic BB rat reported that the expected percentage of animals became diabetic, despite the fact that animals were raised in a sterile environment, which excluded viral and bacterial pathogens. This initially suggested that the BB rat diabetic syndrome is not influenced by infection. However this study was incomplete, as it did not exclude vertically transmitted or slow viruses.

Two subsequent studies (377,378) both showed that inoculation of 30 day old DP-BB rats with lymphocytic choriomeningitis virus could significantly reduce the incidence of diabetes over a prolonged period of time. These rats were normoglycaemic, had normal levels of pancreatic insulin and little or no mononuclear cell infiltration in their pancreatic islets. The infection was primarily lymphotrophic, and pan T cells and cytotoxic/suppressor T lymphocyte subsets were markedly

reduced for 4-7 days after infection. This selective immunosuppression induced by the virus appeared to be enough to suppress the autoimmune response that would have otherwise caused IDDM.

More recently, an outbreak of spontaneous diabetes among DR-BB rats coincided with the serological evidence of the onset of a viral infection (379). The incidence of spontaneous diabetes in the DR-BB rat is normally <1%. This apparent link between viral infection and the expression of diabetes was further investigated using polyionosonic-polycytidic acid (poly I:C; an inducer of interferon known to accelerate diabetic onset in DP-BB rats), and a cytotoxic anti-RT6 moAb, also known to induce diabetes and thyroiditis in DR-BB rats. Neither were effective at inducing diabetes in seronegative DR-BB rats unless used together, whereas either reagent alone was successful when given to seropositive DR-BB rats. Poly I:C also accelerated the onset of DP-BB diabetes to a greater extent in seropositive, as opposed to seronegative, rats. Neither agent was successful when given to non-BB rats. It was therefore suggested that expression of the genetic predisposition to diabetes present in all BB rats depended on cellular factors, including modulatory environmental factors such as viral exposure to pathogens.

Like et al (178) reported that elimination of environmental viruses by caesarean derivation increased the frequency and accelerated the tempo of spontaneous diabetes among DP-BB rats. This effect was not accompanied by any changes in the lymphocyte subsets. The viral-antibody free environment did not alter the resistance of DR-BB rats to diabetes, suggesting that environmental viral pathogens may act to inhibit effector cell function in lymphopenic DP-BB rats, while enhancing effector cell activity in non-lymphopenic DR-BB rats.

Thus, viral infection may affect the expression of diabetes in DP-BB and DR-BB rats in opposite ways, in that the absence of viral pathogens is associated with enhanced susceptibility of DP-BB rats to spontaneous diabetes, but reduced susceptibility of DR-BB rats to induced diabetes. Environmental viruses may protect DP-BB rats by directly infecting and downregulating a subset of peripheral helper T cells, as was observed with the lymphocytic choriomeningitis virus. In contrast, infection of suppressor cells may result in the upregulation of effector cells in DR-BB rats leading to a decreased susceptibility to IDDM. Thus, the different degrees of immunocompetence of DP-BB and DR-BB rats, resulting in different clearance capacities of viral pathogens, may contribute to the differential expression of autoimmune diabetes in virally-infected BB rats.

In addition, chronic viral infection is a common stimulus for the release of interferons from epithelial cells which have been shown to enhance expression of MHC class I molecules by pancreatic endocrine cells *in vitro*. Hyperexpression of MHC class I molecules on pancreatic β-cells is an early and striking feature in prediabetic BB rats, thus indicating that the role of viruses in the pathogenesis of IDDM deserves further attention.

1.3.1.7.2. Non-viral infection

As mentioned earlier, BB rats are also very susceptible to mycoplasmic infections. Mycoplasma are known to be strong mitogens which activate both T and B lymphocytes. It has been reported that such infection resulted in a reduced incidence of IDDM in a BB colony on two seperate occasions (380). Rederivation of the colony led to an increase in the diabetic incidence again, almost to 100%.

1.3.1.7.3. Diet

Early studies demonstrated that the expression of diabetes in the BB rat was not influenced by diet (381). Weanling BB rats placed on either a high carbohydrate, protein or fat diet did not show any significant difference in their incidence of diabetes when followed for 120 days. However, Elliot et al (382) demonstrated that the incidence of IDDM in DP-BB rats fell from a normal 50% down to 15% when the standard chow diet was replaced by a semi-synthetic diet from weaning, in which L-amino acids were substituted for natural intact proteins. When this basic semi-synthetic diet was supplemented with glucose or milk, the incidence of diabetes again rose to 35% and 52% respectively. The presence of intact proteins in the diet therefore appears necessary for the full expression of the genetic susceptibility to develop diabetes in the BB rat. More recently certain plant protein sources found in a cereal-based rodent diet were shown to have an influence on the spontaneous development of diabetes in the BB rat (383). Other studies have also tried to identify dietary components that may have an effect on the incidence of diabetes (summarised by Scott et al, 384). Milk proteins and cereal-based diets were found to have the strongest diabetogenic effect and have been designated " initiators " of disease, and are capable of triggering the events that lead to the eventual destruction of β-cells. Hydrolysed caesin-based purified diets were reported to be the weakest diabetogenic agents (384). Additionally, circulating antibodies to dietary antigens such as bovine serum albumin and crude wheat gliadin may be elevated in the DP-BB rat. The mechanisms by which nutrients in diets protect from diabetes remain unknown (385). No correction of T cell lymphopenia, improvement of T cell proliferative response to mitogens or significant alterations of T cell subset distribution have been observed (386). It should be noted that chemicals or

microbial agents associated with the protein source rather than the protein itself could mediate the observed effects.

Dietary lipids and lard are potent immune system modulators, and the preventive effects of a diet deficient in essential fatty acids in both DP-BB and RT6-depleted DR-BB rats have been demonstrated (387). This effect could be overcome if the animals were given dietary supplements of linoleate beginning at 70 days of age, but not after the peak incidence of diabetes at 120 days of age. Again the mechanisms underlying the protective effect of a fatty acid-free diet are unknown, but it has been suggested that this diet interferes with the generation of lipid mediators such as leukotriene B4 and platelet activating factor (388), both of which may act to attract leukocytes to a focal inflammatory site. Alternatively, the preventive effect may be mediated through an effect on macrophages, as essential fatty acid deficiency prevents the influx of macrophages to injured tissue in glomerulonephritis (389).

Dietary components clearly influence the expression of the diabetic syndrome in the BB rat. It is therefore important to identify the chemical nature of such components before their preventive mode of action can be understood, and perhaps used in a clinical situation.

1.3.1.7.4. Stress

A preliminary study suggested that environmental stress may modify both the age of onset and, less certainly, the incidence of overt diabetes in DP-BB rats (390). Stress applied to animals in this experiment included overcrowding, periods of restraint, slow rotation and resocialisation. However, stress in the form of ultrasound on a short but daily basis from an early age had no effect on the age or onset of diabetes in the BB

rat (391). More recently, Lehman et al (392) subjected 25-day-old DP-BB rats to a triad of stressors, designed to model chronic-to-moderate stress over a 14-week period. Animals were weighed and tested for glycosuria twice weekly and blood glucose measurements performed weekly to 130 days of age. Chronic stress was found to significantly increase the incidence of IDDM : 80% and 70% of the male and female stress animals developed diabetes respectively, compared with 50% in both control groups. Interestingly, stressed males developed manifest diabetes at the same time as their matched controls, whereas stressed females had significantly delayed onset in relation to controls.

Other hormonal and environmental factors, including castration, vagotomy, hypophysectomy, 3-methyl-O-D-glucose (an agent that protects against certain β-cell toxins), and nicotinamide did not affect the cumulative incidence of diabetes (261).

1.3.1.8. PREVENTION OF SPONTANEOUS DIABETES IN THE BB RAT

It has been reported that cyclosporin A (CsA) when given in the prediabetic period prevented the development of IDDM in DP-BB rats to a variable extent. No DP-BB rats receiving oral CsA from 40-150 days of age developed diabetes by an age of 150 days, nor after stopping CsA treatment (393). In a longer-term study using the BB/E rat, CsA administered from 30-100 days of age completely prevented the development of diabetes up to an age of 150 days, and reduced the incidence to 50% compared with controls at 452 days of age (256). Thus, treatment with CsA in the prediabetic period both postponed the onset and reduced the overall incidence of diabetes in DP-BB rats. The appearance of circulating ICSA closely correlated with the development of diabetes, and were not found in any CsA-treated rat up to 150 days of age, but subsequently occured in 79% of CsA-treated and 80% of control rats

developing diabetes by 450 days of age. In contrast, circulating IAA were detected in 16% of CsA-treated rats during treatment and in 15% of diabetic and non-diabetic rats regardless of treatment regimen. Although CsA is thought to exert its immunosuppressive effect specifically by inhibiting the production of messenger RNA for lymphokine production by primed T lymphocytes, in this study the most striking result of CsA treatment was the reduced accumulation of $ED1^+$ macrophages at perivascular or periductal sites adjacent to islets free of infiltration, an early event in the pancreas of the prediabetic BB/E rat. The subsequent recruitment of immune effector cells was also markedly inhibited. The findings that CsA directly inhibited $ED1^+$ macrophage recruitment suggests they may play a key role in the development of insulitis in the DP-BB rat. As anticipated, CsA treatment at diagnosis of IDDM did not ameliorate the disease in the BB/E rat since by this time, the central core of insulin-containing cells has completely disappeared from virtually all the islets throughout the pancreas, leaving only end stage islets consisting of rings or clumps of glucagon-containing cells.

FK506 is another potent immunosuppressive agent that suppresses T lymphocyte activation by inhibiting the transcription of mRNA's for early phase lymphokines, although it is chemically unrelated to CsA . The central action of CsA and FK506 is thought to involve the inhibition of the enzyme peptidylpropyl isomerase, which is the principal constituent of their cytosolic binding site. FK506, when given intragastrically in doses of 1 or 2 mg/kg/day to prediabetic DP-BB rats from 30-120 days of age, reduced the incidence of diabetes from 75% in water-dosed controls to 15% and 0% respectively (394). Animals protected from diabetes by low or high-dose FK506 had normal intraperitoneal (i.p.) glucose tolerance tests, virtual absence histopathologically of autoimmune insulitis, normal liver and kidney function tests and normal pancreatic insulin content.

DP-BB rats receiving weekly i.p. injections of 0.2 mg of OK-432, a non-immunosuppressive streptococcal preparation, starting from 5-6 weeks of age and continuing through to 20-30 weeks of age had a significantly lower cumulative index of IDDM than that found in non-treated DP-BB rats (395). Additionally, the pancreas of OK-432-treated rats retained a greater number of non-infiltrated, intact islets, and the populations of pan, $CD4^+$ and $CD8^+$ peripheral blood lymphocytes were unchanged.

Finally, treatment of DP-BB rats with tetradine, a novel anti-inflammatory plant alkaloid, at a dose of 20mg/kg/day from 35 to 120 days of age also reduced the cumulative index of IDDM from 75.5% to 10.9% (396). The reduction of the incidence of diabetes was not as marked if tetradine treatment was delayed until 70 days of age. Histologically, pancreases from tetradine-treated rats showed a lesser degree of insulitis than controls, and drug toxicity was not observed. Thus, non-immunosuppressive drugs such as OK-432 and tetradine may be of value in preventing diabetes, either alone or in combination with potent immunosuppressive drugs, such as CsA or FK-506, thereby enabling the intermittent use or lowering in dosage of the latter, thus decreasing toxicity without loss of efficacy.

1.3.1.9. CONCLUSION

The diabetic syndrome of the BB rat shares many characteristics of human IDDM. There is a genetic predisposition to diabetes development, and the long prediabetic period is followed by the abrupt onset of symptoms at puberty. Symptoms include weight loss, glycosuria, hyperglycaemia, and hypoinsulinaemia with subsequent ketoacidosis which often proves fatal unless exogenous insulin is administered. The onset of diabetes is accompanied by pancreatic insulitis, similar to that described in

human IDDM, with macrophages, T lymphocytes (both helper and cytotoxic/suppressor subsets), NK cells and B lymphocytes present at the onset of diabetes.

The inheritance of diabetes in the BB rat remains unclear. Development of disease requires the presence of one MHC complex-associated gene, and at least two additional genes located outside the MHC region. Both cellular and humoral immunity is involved in the pathogenesis of diabetes in the BB rat as evidenced by adoptive transfer experiments, autoantibodies to islet cell components, the presence of pancreatic insulitis, and successful immune intervention studies.

However, differences in characteristics between human and BB rat IDDM have been reported, particularly the absence of ICCA and the presence of lymphopenia and T cell defects in diabetic BB rats. It should be noted that BB rats can develop diabetes in the absence of lymphopenia and/or depressed T cell responses, suggesting that neither abnormality is essential for the development of diabetes in this animal model.

1.3.2. **THE NON-OBESE DIABETIC MOUSE**

1.3.2.1. INTRODUCTION

In 1974, a female mouse in the normoglycaemic subline of Jcl-ICR mice was found to be overtly diabetic, and the non-obese diabetic (NOD) mouse strain was subsequently established by Makino and co-workers of the Shionogi Research Laboratories in Aburahi, Japan (397,398). The mice were found to have many characteristics similar to human autoimmune diabetes and have increasingly been used as an animal model in the study of IDDM (397). The incidence of spontaneous

diabetes is relatively constant after many generations of selective breeding, and is influenced by gonadal sex hormones. By 30 weeks of age, 70-80% of female and approximately 20% of male NOD mice become overtly diabetic (399), characterised by polydipsia, polyuria, hyperglycaemia, glycosuria and, in some mice, ketonuria. Female NOD mice castrated at 5 weeks of age showed an incidence of diabetes as low as that of untreated males, whereas castration of males increased the incidence of overt disease to that of non-operated female mice (400). A similar experiment showed that the removal of sex organs at 10 weeks of age did not affect the incidence of diabetes in either sex. However, no NOD mice of either sex developed diabetes upon testosterone administration, whereas oestradiol administration caused the appearance of overt diabetes in both sexes of mice, directly demonstrating that sex hormones regulate the development of diabetes in NOD mice.

1.3.2.2. PATHOGENESIS

Diabetes development in NOD mice is histologically characterised by a leukocytic infiltration of the pancreatic islets. A pervasive insulitis emanating from the pancreatic vasculature and secretory ducts is first observed at a time when the islets are free of lesions. Consequently, leukocytes aggregate at the periphery of islets (peri-insulitis) prior to penetrating the islet core. This widespread insulitis is evident between 4 and 7 weeks of age (401), and is virtually complete at the clinical onset of diabetic symptoms (approximately 12-15 weeks of age). At this time marked decreases in pancreatic insulin content are observed (402). Onset of diabetes is marked by the appearance of moderate glycosuria and non-fasting plasma glucose higher than 13.9 mmol/l. Both glycosuria and hyperglycaemia become progressively more severe over the following 3-4 weeks, during which weight loss, polydipsia and polyuria are observed. In contrast to the destruction of insulin producing β-cells leading to

hypoinsulinaemia and the onset of clinically overt diabetes (403), other islet endocrine cells are untouched by the immune response. Without exogenous insulin treatment, NOD mice become severely hyperglycaemic and ketonaemic, but not ketoacidotic (404).

1.3.2.3. IMMUNOPATHOGENESIS

The subsets of mononuclear cells infiltrating the pancreatic islets in the prediabetic NOD autoimmune process have been determined by immunohistochemical analysis. The predominant cell type was found to be activated T lymphocytes, including helper/inducer $L3T4^+$ and cytotoxic/suppressor $Lyt\text{-}1^+$. $Lyt\text{-}2^+$ T cells were less frequently encountered (405). These observations suggest that diabetogenesis is a T lymphocyte dependent process. B lymphocytes and macrophages are also present in the early islet infiltrate (406,407), whereas NK cells are functionally defective (408). Additional evidence for an autoimmune aetiology in the NOD mouse came from Wicker et al (409) who developed an adoptive transfer protocol that used splenocytes from overtly diabetic NOD mice to effectively transfer diabetes to >95% irradiated NOD mice that were >6 weeks of age, within 12-22 days, i.e. at a higher frequency and at a younger age. Transfers were unsuccessful in recipient NOD mice <6 weeks of age. Furthermore, even though male NOD mice display a lower incidence of spontaneous diabetes than the female, transfers into males and females were equally successful.

Hanafusa et al (410) further identified the T lymphocyte subset responsible for the transfer of insulitis or diabetes in T cell depleted NOD mice which rarely develop insulitis. Spleen and lymph node cells isolated from diabetic mice treated with cyclophosphamide (a compound known to significantly increase the incidence of

diabetes at an age when spontaneous diabetes rarely occurs in untreated NOD mice) were separated into various T lymphocyte subsets using moAb against various surface antigens in conjunction with complement. These subsets were then adoptively transferred to NOD mice recipients. Transfer of either the Thy-1^+, Lyt-1^+ or L3T4^+ cell-depleted fraction of T cells caused no increase in the incidence of insulitis in T cell depleted NOD mice. In contrast, donor cells depleted of the Lyt-2^+ fraction successfully induced insulitis in T cell depleted recipient mice. These data were consistent with immunohistochemical studies which showed that 2 weeks after cell transfer, L3T4^+ helper T cells were the predominant phenotype of cells infiltrating the islets, again indicating the importance of the L3T4^+Lyt-2^- T lymphocyte subset in the pathogenesis of insulitis in NOD mice.

More recently, Christianson et al (411) performed transfer studies in NOD mice homozygous for the severe combined immune deficiency (scid) mutation, which fail to develop functional T and B lymphocytes and are consequently insulitis- and diabetes-free. Purified splenic L3T4^+, but not Lyt-1^+, T cells from diabetic NOD donors already primed to β-cell antigens adoptively transferred insulitis and diabetes into unirradiated NOD-scid/scid recipients. However, purified splenic L3T4^+ T cells from prediabetic NOD mice were incapable of transferring disease in the absence of Lyt-1^+ T lymphocytes, confirming that these T cells are required for the initiation of β-cell destruction during the natural course of the disease in euthymic NOD mice (411).

These findings are in agreement with Koike et al (412) who reported that NOD neonatal mice treated with injections of the anti-L3T4 moAb GK1.5 for 12 weeks prevented the insulitis and diabetes associated with NOD mice. At the same time, Wang et al (413) also reported success with this moAb in the prolonged survival of

cultured BALB/c mice islets allografted into GK1.5-treated diabetic NOD mice. Treatment reduced the level of $L3T4^+$ T lymphocytes from an initial 43% of the peripheral blood lymphocytes to <4%, and levels remained low for approximately two weeks after cessation of GK1.5 treatment. The gradual return to hyperglycaemia roughly correlated with the reappearance of $L3T4^+$ T lymphocytes in the peripheral circulation.

Thus, it is widely agreed that class II-restricted $L3T4^+$ T cells play an essential role in the development of diabetes in the NOD mouse. However, two studies have reported that for the successful transfer of diabetes to NOD neonatal mice using spleen cells from diabetic NOD mice donors, both $L3T4^+$ and $Lyt\text{-}2^+$ T cells are necessary (414,415). This finding suggests that disease transfer probably involves cooperation between these T cell subsets in the effector phase of β-cell destruction. Neither insulitis nor diabetes was induced in young irradiated recipients by transfer of either T cell subset alone, and it is likely that $L3T4^+$ cells act as helper cells cooperating with activated $Lyt\text{-}2^+$ cytotoxic cells, possibly by providing expansion signals such as secretion of the cytokine, IL-2. Miller et al (414) further demonstrated that both T cell populations had to be obtained from diabetic NOD mice rather than from young non-diabetic donors, in order to successfully transfer diabetes and/or insulitis to the NOD recipient.

Most recently, Wicker et al (416) in an attempt to assess the contribution of cytotoxic/suppressor $Lyt\text{-}2^+$ T cells to diabetes developed a class of NOD mouse that lacks expression of β2-microglobulin (NOD-B2mnull). These mice do not express cell surface MHC class I molecules or produce detectable levels of $Lyt\text{-}2^+$ T cells in the periphery, and fail to develop insulitis and diabetes. The observation that NOD-B2mnull mice do not develop $L3T4^+$ T cell mediated insulitis suggests that at

least one role of Lyt-2^+ T cells is at the initiation of the autoimmune response to β-cells in the NOD mouse. Serreze et al (417) confirmed these findings in the MHC class I deficient NOD-B2mnull mouse, and also reported that the presence of a disrupted B2m locus did not negatively affect plasma insulin levels in these mice.

It has been demonstrated by a number of groups that cells can process and present peptide antigens derived from intracellular sources in the context of their own MHC class I molecules (418). $CD8^+$ T cell lines from NOD mice which recognise antigens in the context of MHC class I molecules are able to kill islet cells in an MHC-restricted manner (419). 70% of lymphocytes derived from the culture of cells isolated from the insulitis lesions of the diabetic NOD pancreas in the presence of IL-2 were Lyt-2^+, and were capable of killing islets, but not thyroid cells, in a manner restricted by the MHC haplotype of target islet cells. These results suggest that islet cells are able to present islet-specific peptide antigen to autoreactive T cells that mediate specific killing. In contrast, MHC class II antigens are not normally expressed on islet β-cells, and the ability of β-cells to present antigens to $CD4^+$ T cells in the context of MHC class II has not been demonstrated. $L3T4^+$ helper T cells in NOD mice are stimulated by β-cell antigen(s) expressed on, or presented by, class II bearing cells, such as macrophages and dendritic cells, to produce lymphokines. Recently, a $L3T4^+$ T cell line cloned from a NOD mouse was shown to proliferate and produce lymphokines in response to islet cell antigen and NOD mice APC (420). This cell line was shown to destroy grafted islet cells in a tissue-specific manner. The secreted lymphokines activate class I restricted Lyt-2^+ T cells specific for the same β-cell antigen, and proceed to mediate β-cell destruction. Lyt-2^+ T lymphocytes were shown to inhibit insulin release from normal islet cells in response to theophylline/arginine stimulation (421).

Support for a class I restricted effector mechanism came from transplantation studies in the NOD mouse (H-2K^d , D^b) (422). In these studies pancreases from newborn histoincompatible CBA (H-2^k) mice were accepted by CsA-treated diabetic NOD mice. However, islets within the donated pancreases were rejected, suggesting that CsA treatment at 40mg/kg/day could not prevent the pre-existing anti-β-cell autoimmunity. Islets isolated from BALB/c and B.10 mice, both of which share class I antigen with the NOD mouse (K^d and D^b respectively), showed a significant increase in lymphocytic infiltration compared with CBA islets. This suggests that effector cells utilise class I antigen as restricting elements for the autoimmune response, and anti-islet autoimmunity in NOD mice appears to exert its effect in an H-2 restricted manner. In contrast, Prowse et al (423) cultured CBA islets in order to remove APC that may cause allograft rejection before transplanting them into NOD mice. These CBA grafts were still rejected despite the acceptance of CBA cultured pituitary tissue transplanted to the same NOD mice. This group hypothesised that an MHC-unrestricted mechanism was responsible for the β-cell destruction seen in the NOD mouse. However, these results may not have been due to the effect of MHC antigens alone, as diverse minor alloantigens are also present in these donor mice. Immune responses to minor alloantigens also involve MHC antigens and may influence islet allograft destruction. Recently, this group repeated their previous studies in the absence of minor alloantigenic differences between donor mouse strains (424). The pancreata from three B.10 congenic lines were transplanted under the kidney capsule of NOD mice immunosuppressed with a dose of CsA that prevents allorejection, but not autoimmune destruction, within one month of diabetes onset. All grafts were harvested ten days after transplantation and examined immunohistologically. Pancreatic allografts from B.10 congenic mice sharing H-2K^d with NOD mice showed the strongest infiltration, and no islets or β-cells were evident. Pancreases from mice sharing the same H-2D^b also showed severe

lymphocytic infiltration and only a few β-cells were present as single cells. In contrast, grafts completely incompatible at the H-2 locus showed the least infiltration, and normal islets containing many β-cells were found in the majority of grafts. These results confirm the previous findings of this group, i.e. islet allograft destruction in diabetic NOD mice is MHC-restricted and not controlled by CsA therapy. In a seperate study, Matsuo et al (425) reported the presence of non-MHC-restricted islet allograft damage which was milder in its anti-islet response and easily controlled by CsA.

These results demonstrate the involvement of other mechanisms in β-cell destruction, such as macrophage-mediated production of oxygen free radicals which can damage islet β-cells (426). The contribution of oxygen free radicals to the development of insulitis in NOD mice was examined by administration of the scavengers superoxide dismutase and catalase. Treatment with superoxide dismutase reduced the number of islets with insulitis compared with the control group, and catalase treatment gave similar, although non-significant results. The content of reactive oxygen intermediates in islet cells in a culture system were measured by flow cytometry and the effect of peritoneal exudate cells and T cells on their production were examined. Peritoneal exudate cells, but not T cells, from NOD mice increased the content of reactive oxygen intermediates in islet cells of NOD mice. The addition of superoxide dismutase to the culture medium suppressed this increase, thereby supporting the concept that production of oxygen free radicals mediated by macrophages can damage islet β-cells, directly resulting in autoimmune diabetes in NOD mice.

As mentioned earlier, macrophages also play an important role in the initiation of insulitis in the NOD mouse and are required to recruit or activate diabetogenic T lymphocytes (427,428). Silica is selectively toxic to macrophages and i.p.

injections completely prevented the development of insulitis and diabetes in NOD mice, either treated with cyclophosphamide or untreated. These findings were confirmed by Charlton et al (429), who additionally reported that administration of anti-Lyt-2^+ moAb into cyclophosphamide-treated NOD mice prevented the occurrence of diabetes, demonstrating that both Lyt-2^+ T lymphocytes and macrophages were necessary, but not sufficient, for β-cell destruction in the NOD mouse. This group therefore proposed that macrophages present β-cell antigen to L3T4^+ T cells which induce cytotoxic Lyt-2^+ T lymphocytes to specifically destroy β-cells.

Yasunami and Bach (430) also used cyclophosphamide-induced diabetic NOD mice for their studies. Spleen T cells from these mice were shown to be capable of transferring diabetes into irradiated non-diabetic syngeneic recipients, indicating that the diabetogenic effect of cyclophosphamide is not mediated by direct toxicity on pancreatic β-cells, but by abrogation of a suppressor mechanism which may prevent activation of T cells responsible for the development of diabetes in the NOD mouse. The importance of abnormal suppressor function has also been reported by Serreze and Leiter (408) who found a defect in suppressor cell activation rather than the absence of this immunoregulatory cell population in NOD mice after reporting a T cell response to self-MHC class II antigens in a syngeneic mixed lymphocyte reaction (MLR).

Several groups have suggested the presence of haematopoietic stem cells in the bone marrow of NOD mice, which may be the site of origin of these immunological abnormalities. Ikehera et al (431) reported that when 6 month old NOD mice were irradiated and reconstituted with bone marrow cells from young (<2 month old) BALB/c nu/nu mice, the NOD mouse developed neither insulitis nor overt

diabetes and had normal T and B cell functions. In addition, the newly developed T cells were tolerant to cells with both donor and host-type MHC determinants. A similar result was reported by Wicker et al (432) who constructed radiation bone marrow chimeras in which (NOD x B.10) F_1 irradiated recipients were reconstituted with NOD bone marrow cells. Insulitis was observed in the majority of F_1 chimeras and 31% developed diabetes. In contrast, unmanipulated (NOD x B.10) F_1 mice or irradiated F_1 mice reconstituted with F_1 or B.10 bone marrow did not develop insulitis or diabetes. These data further demonstrate that expression of the diabetic phenotype in the NOD mouse is dependent on NOD-derived haematopoietic stem cells. Furthermore, since F_1 chimeras reject β-cells within pancreas transplants from newborn B.10 mice, the β-cells of the NOD mouse do not appear to express a unique antigenic determinant that is the target of autoimmune response. Finally, Serreze et al (433) observed autoimmune β-cell destruction in lethally irradiated diabetes-resistant F_1 mice from an outcross between NOD and non-obese normal inbred strains after adoptive transfer of haematopoietic stem cells from NOD donors. These defects have been attributed to APC, such as macrophages and dendritic cells that develop from myelocytic precursors in the bone marrow. In contrast, F_1 mice reconstituted with either non-obese normal or F_1 bone marrow did not develop hyperglycaemia.

1.3.2.3.1. Humoral immunity

The presence of various autoantibodies in the sera of NOD mice has been reported by several groups. Reddy et al (434) examined the ontogeny of insulitis, ICA, and IAA cross-sectionally at days 15, 25, 40 and 90 in young female NOD mice. ICA and IAA were studied longitudinally from day 35 or day 144-168, until day 250. Insulitis was first observed at day 40 (50%) and subsequently at day 90 (70%). ICA and IAA were present at day 15 in 46% and 54% of the animals respectively. The incidence of

ICA was slightly higher at day 25 (60%) than at day 40 (40%) and day 90 (54%), whereas IAA were present in all samples from days 25-90. At day 40 and 90, insulitis and IAA were present together in 42% and 70% of the animals respectively, whereas insulitis and ICA had a lower rate of concordance : 17% and 42% respectively. The concordance rates for ICA and IAA were 42% at day 40 and 54% at day 90. Concordance for all three markers was first observed at day 40 (17%) which increased to 38% at day 90. In longitudinal studies, ICA and IAA were often present together whether diabetes supervened or not. The three markers investigated are expressed early after birth and well before clinical symptoms appear in NOD mice, but their early expression alone is not predictive of diabetes. Using an ELISA assay, Pontesilli et al (435) detected IAA in all NOD mice throughout the entire period of study. Autoantibodies reacting with the cytoplasm of islet cells in Bouins fixed pancreas sections were found in 47%-58% of the samples from NOD mice aged 75-150 days. The percentage of circulating T lymphocytes and their $L3T4^+$ and $Lyt\text{-}2^+$ subsets were also found to be higher in NOD mice for all ages and both sexes than in controls. However, neither the autoantibodies or lymphocyte abnormalities are predictive of the appearance of overt diabetes.

Yokono et al (436) demonstrated the presence of circulating autoantibodies to ICSA in some NOD mice by a protein-A ligand assay after establishing the moAb to ICSA from NOD splenocytes.

Atkinson and MacLaren (437) reported that the sera from newly diagnosed NOD mice contained an autoantibody to the 64 kD β-cell protein, GAD. The autoantibody was detectable at weaning but disappeared within weeks of diabetes onset, and was absent from older non-diabetic NOD mice as well as several non-diabetic control mice strains.

The T cell reactivity to an antigen cross-reactive with a mycobacterial 64 kD heat shock protein is also thought to be associated with the pathogenesis of diabetes in the NOD mouse (438). The specificities of the helper T cell clones capable of causing hyperglycaemia and diabetes were studied in order to identify the critical peptide epitopes. A peptide within the sequence of the human variant of the 65 kD heat shock protein was discovered. T cell clones recognising this functionally important peptide mediate insulitis and hyperglycaemia and administration of the peptide itself to NOD mice was found to downregulate immunity to the 65 kD heat shock protein and prevent the development of diabetes.

Recently, Gelber et al (439) attempted to identify the earliest T cell autoantigen in the NOD mouse, reasoning that this antigen could be casually involved in the initiation of the disease. To this end the spontaneous T cell proliferative response of peripheral lymphatic tissue from young prediabetic NOD mice to extracts of a pancreatic insulinoma cell line were determined. T cell proliferative responses to whole cell extracts and subcellular fractions of the insulinoma were seen in NOD mice >8 days old, demonstrating that T cells responsive to β-cell antigens exist in the peripheral lymphoid tissue of these mice before manifestation of histologically detectable insulitis. T cell clones derived from young, prediabetic NOD mice also responded to whole cell extracts and the subcellular fractions of the insulinoma as did peripheral lymphocytes. However, the clones did not respond to purified autoantigens, including GAD 65 or 67, ICA69, carboxypeptidase-H, peripherin, insulin or 65 kD heat shock protein, suggesting that these responses were specific for novel β-cell autoantigens. Finally, NOD T cell proliferative responses were also observed to an extract of human islets, suggesting potential shared antigenic determinants between human and mouse β-cells. If the human and murine β-cell-specific antigens are conserved, the identification of these murine

autoantigens may lead to the discovery of the human homologue, thus leading to possible immunotherapy and/or early diagnosis of IDDM.

Other autoantibodies have also been reported in NOD mice (440), e.g. antibodies reacting with the apical border of the thyroid follicular cells or with the duct cells of submandibular glands. Older NOD mice have anti-nuclear antibodies. However, sera from NOD mice do not contain antibodies against adrenal glands or gastric parietal cells regardless of age.

1.3.2.4. GENETICS

The susceptibility of NOD mice to autoimmune IDDM is under complex polygenic control, with environmental factors exerting strong effects on gene penetrances. Genetic analyses (398,441,442) have shown that at least 3 recessive genes are involved, the most important of which is tightly linked to the MHC genes on chromosome 17, i.e. H-$2g^7$. Sequence analysis of the MHC genes and their corresponding molecules by Acha-Orbea and McDevitt (443) has shown that unique genetic abnormalities in the class II MHC region contribute to the propensity of the NOD mouse to develop diabetes. Mouse MHC genes encode two types of class II proteins : I-A and I-E. The I-A molecule is involved in the recognition of antigens by T cells, whereas the I-E protein plays an important role in normal suppressor activity. The nucleotide sequence of I-A molecules in the NOD mouse is different from any known I-Aβ gene, resulting in the expression of a unique class II I-Aβ molecule. Furthermore, although the class II I-Eα gene is present, mRNA for this gene is absent, resulting in a complete lack of expression of I-Eα molecules. These genes may therefore be directly responsible for the development of insulitis and/or diabetes in NOD mice.

Nishimoto et al (444) used backcross studies to further investigate the contribution of the I-Eα defect to insulitis occurrence in the NOD mouse. The F_1 mice of a cross between I-Eα deficient mice and C57BL mice expressing the I-Eα molecule were crossed with NOD mice. Offspring expressed I-Eα molecules, and the development of autoimmune insulitis was prevented. This group also reported that the I-E gene directly injected into NOD mice induced the expression of I-E molecules in recipients, and again insulitis failed to develop. These data highlight the important contributory role that the I-Eα defect plays in the occurrence of insulitis. However, although the diabetogenic H-$2g^7$ haplotype is permissive for insulitis development, insulitis sufficiently widespread to produce the clinical phenotype of diabetes is rarely observed in segregants that are not homozygous for the diabetogenic class II MHC alleles.

The mechanism leading to the prevention of insulitis in I-E bearing NOD mice is unknown. Possible mechanisms include the induction of suppressor T cells as a result of I-E expression, which prevent the generation of autoreactive β-cell-specific helper T cells. Alternatively, I-E molecules may crossreact with an epitope formed by interaction of the β-cell antigen and MHC molecules. Thus, the expression of I-E in the early neonatal stage can make T cells tolerant to autologous β-cells. However, the lack of the I-Eα gene is not the sole cause of diabetes, since many otherwise normal strains of mice not expressing I-E proteins do not develop insulitis or diabetes.

As observed in the human and spontaneously diabetic BB rat, an aspartic acid residue at position 57 of the NOD mouse I-Aβ chain (homologous to human HLA-DQβ chain) contributes to the protection of islet β-cells against an autoimmune response. The substitution of aspartate at this position with serine, as found in the NOD mouse, may alter the 3-D configuration of the I-Aβ molecules resulting in the aberrant

recognition of the β-cell autoantigen by T lymphocytes, which finally leads to the development of an autoimmune reaction against the islet β-cell.

Taken together, these results suggest that either the lack of I-Eα expression or the unique amino acid sequence of the I-Aβ chain could be responsible for the development of diabetes in the NOD mouse. Roles for 2 out of the 3 recessive diabetogenic genes have also been proposed. The second diabetogenic gene, which is located close to the gene for the T cell surface antigen Thy-1 on chromosome 9 (445,446), may play a primary role in the development of insulitis. Subsequently, the first MHC-linked diabetogenic gene might contribute to the progression from insulitis to overt diabetes. No information is presently available on the third diabetogenic gene.

Although immunogenetic analyses has concentrated on the diabetogenic contributions of the MHC class II region of the H-$2g^7$ haplotype, current evidence suggests that the haplotype as a whole should be considered as contributing to diabetes susceptibility. Strong evidence comes from the congenic transfer of the haplotype of a NOD mouse-related strain, cataract Shionogi, which contains the same class II alleles but distinct class I loci, onto the NOD inbred background, resulting in a lower incidence of diabetes and insulitis in the mice homozygous for the cataract Shionogi MHC (398,447). Intra-MHC regions both proximal and distal to the H-$2g^7$ class II region therefore contain alleles that may contribute to diabetes susceptibility, including a unique heat-shock protein 70 allele (448) and alleles at *Tap-1* and *Tap-2* (449). The products of the *Tap-1* and *Tap-2* loci are members of ATP-dependent transport proteins, and may function to transport processed antigenic peptide fragments generated in endosomal compartments into the lumen of the endoplasmic reticulum for association with MHC class I molecules (450). Defective *Tap*-gene transcription

in NOD mice spleens has recently been reported (451), but not in cultured peritoneal macrophages (452).

Non-MHC genes also control diabetes development, and analysis of MHC congenic stocks has shown that the NOD strain diabetogenic H-$2g^7$ haplotype requires interaction with non-MHC linked susceptibility to mediate IDDM (453).

1.3.2.5 ENVIRONMENTAL FACTORS

Diabetes in the NOD mouse has been categorised as an autoimmune disease based on the involvement of various effector systems, including macrophages, T lymphocytes, (particularly class II-restricted $L3T4^+$ and class I-restricted Lyt-2^+ T lymphocytes), and humoral mediators. Environmental factors are also important modulators of genetic susceptibility to IDDM, and account for a major component of the variation observed in the incidence of diabetes in NOD mice among different colonies (441).

1.3.2.5.1. Viral infection

The initial events that trigger the autoimmune response in NOD mice remain largely unknown. It has been suggested that diabetes could result from an immune response to viral antigens in the host cells, or to host cell-specific antigens that are exposed as a result of viral infection. Indeed, Suenaga and Yoon (454) frequently found clusters of endogenous retrovirus like particles in the β-cells of male NOD mice receiving silica for the preservation of islets, and subsequently cyclophosphamide to produce overt diabetes. In contrast, retrovirus-like particles were rarely found in the β-cells of male NOD mice receiving silica alone. Other endocrine cells, including α- and δ-cells,

pancreatic polypeptide-producing cells, and exocrine acinar cells, did not contain such virus-like particles. The clear correlation between the presence of retrovirus-like particles in β-cells and insulitis lesions in the cyclophosphamide-treated mice led to the conclusion that β-cell-specific expression of endogenous retrovirus-like particles is associated with the development of insulitis and diabetes in NOD mice.

However, viral infections are more frequently reported to reduce rather than exacerbate the incidence of diabetes in the NOD mouse. Transfer of male NOD mice from a conventional mouse room into germfree conditions increased the diabetic incidence from 6% to 70% (455). Certain peripheral immunoregulatory functions appear to be defective in NOD mice maintained in specific pathogen-free environments, including defective T-suppressor cell functions *in vitro*, and defects in the differentiation and maturation of APC developing from bone marrow progenitors (456). These defects appear to perturb presentation of self antigens in the course of tolerance induction and are associated with defective secretion of endogenous cytokines, including IL-1, IL-2 and interleukin-4 (457,458). It has been proposed that exposure to viral pathogens counterbalance these defects via elevation of inflammatory cytokine levels. Indeed, exposure of newborn or adult NOD mice to a variety of exogenous murine viruses, e.g. encephalomyocarditis, lymphocytic choriomeningitis and murine hepatitis virus, have been reported to reduce the incidence of diabetes (459,460). Treatment of prediabetic NOD mice with various types of exogenous immunomodulators, including complete Freunds adjuvant (461) also circumvent the development of diabetes (441). Finally, lymphocytes from virus-infected donors failed to transfer diabetes, providing further evidence that these infectious agents protect by providing general immunostimulation. Alternatively, infection may modulate pre-existing autoimmune responses to β-cells, thereby

decreasing the immunological attack against the islet cells and ameliorating the development of diabetes.

1.3.2.5.2. Diet

Elliot et al (462) fed female NOD mice diets containing different proteins from just before weaning. Only mice receiving meat meal or casein as the protein source developed diabetes at the rate expected from this colony. Lactalbumin and gluten did not precipitate diabetes except in a small number of animals. Casein hydrolysate in lieu of protein protects NOD mice against overt diabetes, but only if introduced at an early age. The animals which did not show overt diabetes nevertheless had intermittent trace glycosuria and the majority showed mild degrees of peri-insular lymphocyte infiltration. Both cows milk casein and some unidentified substance in commercial mouse chow therefore appear to be dietary triggers of diabetes in the NOD mouse when introduced at weaning. Coleman et al (463) also reported diabetogenic catalysts present in natural ingredient diets, which contain lipoidal moieties that are absent or present in low concentration in semi-purified diets.

1.3.2.6. PREVENTION OF SPONTANEOUS DIABETES IN THE NOD MOUSE

Many therapeutic interventions can successfully alter the incidence of insulitis and diabetes in the NOD mouse, and these strategies have recently been reviewed by Bowman et al (464). Ogawa et al (465) initially demonstrated that neonatal thymectomy prevented the occurrence of diabetes in NOD mice, further supporting the involvement of T lymphocytes in disease development. Many studies have examined the ability of CsA to suppress and/or ameliorate IDDM in NOD mice. Kida et al (466) reported that oral administration of 20 or 40 mg/kg/day of CsA for 5

weeks from an age of 4 weeks reduced the incidence of insulitis in NOD mice. This was more marked in male rather than female mice. Mori et al (467) treated NOD mice aged 30-60 days with oral doses of 2.5, 15 and 25 mg/kg CsA on alternate days until 160 days of age. This regimen prevented the development of insulitis and diabetes in NOD mice, and histologically the islets of the mice whose diabetes had been prevented with CsA had an almost normal appearance. However, CsA appeared to have little therapeutic effect on diabetes after development of glucose intolerance in NOD mice, even at the higher dose.

Wang et al (468) reported similar findings, and reasoned that since CsA did not inhibit the synthesis of lymphokine once the message is present in the cell, it might be expected that CsA was relatively inefficient as an agent for the regulation of disease recurrence following administration to established diabetic recipients. Furthermore, disease recurrence in cultured islet grafts was found to be a $CD4^+$ T cell-dependent process as determined by the *in vivo* administration of anti-CD4 and anti-CD8 moAb prior to the grafting of islet tissue. However, $CD4^+$ T cell depleted NOD mice transplanted with islet tissue, prior to administration of low-dose CsA therapy still failed to maintain their grafts.

The preventive and therapeutic effects of large-dose nicotinamide administration on diabetes in young female NOD mice has also been studied (469). After 40 days of treatment, all mice showed almost normal glucose tolerance and only mild insulitis upon histological examination. Of the NOD mice given nicotinamide from the day of the first appearance of glycosuria, 66% showed an improvement in glucose tolerance and a disappearance of glycosuria. However, if nicotinamide treatment was withheld until 1-2 weeks after the onset of marked glycosuria, the majority of treated NOD mice did not become negative for urinary glucose, suggesting that nicotinamide

administration only reverses β-cell damage in the early stages of diabetes. It should be noted that nicotinamide may increase intracellular NAD content not only by serving as a precursor in NAD synthesis, but also by possibly reducing poly- and/or mono-ADP ribosylation.

Nomikos et al (365) prevented the autoimmune destruction of allogeneic islet tissue transplanted to NOD mice by treating recipients with combined desferrioxamine and nicotinamide, an iron chelator and inhibitor of hydroxy radical formation. Transplanted animals became normoglycaemic and remained so for the duration of the treatment, suggesting that oxygen-derived free-radicals produced by activated inflammatory cells may be involved in islet damage in the NOD mouse. This group (470) further demonstrated that *in vivo* treatment of NOD mice with the enzyme superoxide dismutase and catalase protected islet tissue from disease recurrence following transplantation into diabetic mice. Similar results were obtained when animals were treated with either enzyme alone. This effect was dose-dependent and little protection was observed when lower doses of enzyme were used. These results indicate that oxygen metabolites, especially superoxide and hydrogen peroxide, are directly involved in the pathogenesis of IDDM in the NOD mouse. Free-radical damage may account for the specificity of β-cell destruction in autoimmune diabetes resulting from superoxide production, as the β-cell is particularly sensitive to free-radical damage.

The mechanism of nicotinamide action has been postulated by Nakajima et al (471). Firstly, nicotinamide was shown to inhibit ADCC activity and thus may modulate immune responses. Secondly, nicotinamide might be utilised to produce NAD, which is reduced in islets of NOD mice. Alternatively, nicotinamide may act as an inhibitor of poly (ADP-ribose) synthetase (472), thus preventing the reduction of NAD and

the breakdown of DNA at the same time. Nicotinamide was also found to prevent the earlier and increased induction of diabetes in NOD mice after cyclophosphamide treatment (473), and ADCC might play a role in the insulitis process. ADCC of splenic mononuclear cells was measured in female NOD mice and age-matched non-diabetes-prone controls. No significant difference in activities was observed between the two groups of prediabetic mice. However, ADCC activities in the diabetic NOD mouse were significantly higher than those in control mice, suggesting that nicotinamide strongly inhibits ADCC of mononuclear cells isolated from diabetic NOD mice.

1.3.2.7. CONCLUSION

Many key features of human IDDM are reflected in the NOD mouse, including the development of insulitis, the inheritance of particular MHC class II alleles representing the major component of genetic susceptibility, the transfer of IDDM by haematopoietic cells in bone marrow and the T cell dependence of disease pathogenesis. However, there are also several important differences between human and mouse disease. The presence of a high percentage of T cells (both $CD4^+$ and $CD8^+$ subsets) in NOD lymphoid tissues and peripheral blood distinguishes NOD mice from human diabetes and also from the severely T lymphopenic BB rat. NOD mice do not display the severe diabetic ketoacidosis characteristic of untreated human IDDM patients, perhaps due to an enhanced ability of mice to metabolise blood ketones to lactate. NOD mice also show a pronounced female gender bias for disease susceptibility that is not observed in humans or BB rats. In addition, the pathogenesis of IDDM in NOD mice is associated with expression of endogenous defective retroviruses in β-cells, a feature that has not been previously described in the β-cells of insulitic islets from BB rats or humans. Finally, many studies use

cyclophosphamide to induce and increase the early onset of diabetes in the NOD mouse. This, in theory, is a chemically-induced animal model of IDDM, rather than a spontaneously autoimmune diabetic model.

1.3.3. THE STREPTOZOTOCIN-INDUCED DIABETIC MOUSE

Streptozotocin is a naturally occurring antibiotic produced by Streptomyces *achromogenes* (474), which was originally screened for use in cancer chemotherapy or as an antibiotic in 1960. During preclinical screening three years later, STZ was found to be diabetogenic in rats and dogs (475), and subsequently in a variety of laboratory animals. Its structure has been determined to be the nitrosamide methylnitrosurea linked to D-glucose. The glucose moiety appears to be the essential component in STZ that specifically directs it to the β-cell, as methylnitrosurea alone is much less toxic to islet insulin-secreting cells (476). Once inside the cell, STZ spontaneously decomposes to form an isocyanate compound and a methyldiazohydroxide (477), which further decomposes to form a carbonium ion. This highly reactive ion is able to alkylate various cellular components, including DNA, leading to lesions which are removed by excision repair. Part of this repair process involves the activation of the enzyme poly (ADP-ribose) synthetase to form poly (ADP-ribose) using NAD as a substrate (476,478). In the β-cell, this enzyme is overactivated and NAD is critically depleted, resulting in a cessation of cellular function and ultimately cell death (479). Indeed, treatment of islets with nicotinamide prevents depletion of NAD and protects against the diabetogenic effect of STZ (480). Although this hypothesis is widely accepted, more recent studies suggest that the mode of action of STZ may involve a more complex mechanism than the overactivation of a single enzyme (476,478).

Various forms of diabetes can be induced by STZ depending upon the administration protocol employed. IDDM occurred following injection of STZ either as a single high dose (>50-60 mg/kg/body weight) or as five consecutive subdiabetogenic injections (40 mg/kg), with the latter leading to a gradual development of permanent, stable hyperglycaemia (481).

1.3.3.1. MULTIPLE LOW-DOSE STZ-DIABETES

Prior to the discovery of the BB rat and NOD mouse, the multiple low-dose STZ diabetic mouse, as developed by Like and Rossini (481), provided researchers with an animal model of diabetes in which β-cell pathogenesis could be observed. Progressively severe glucose intolerance was noted within a few days of the last injection of STZ, and by day 11, heavy insulitis and disruption of islet architecture was observed, eventually leading to permanent, severe diabetes. Interestingly, only males appeared susceptible to diabetes induction (482). Castration of males blunted the level of multiple low-dose STZ-diabetic hyperglycaemia, whereas testosterone treatment restored full sensitivity. Additionally, testosterone treatment of both castrated and non-castrated female mice increased diabetes to levels comparable with those observed in male mice.

IDDM pathogenesis in the low-dose STZ-diabetic model is assumed to involve thymus-dependent immunity against β-cells, possibly mediated by cytotoxic T lymphocytes. Indeed, lethal irradiation eliminated sensitivity of athymic male mice to STZ, whereas sensitivity was restored by reconstitution of T cell enriched splenocytes (483), demonstrating the diabetogenic potential of T lymphocytes in this model.

Diabetes in this multiple low-dose model is not solely due to the direct toxicity of STZ, since microscopic studies revealed mononuclear cells in and around the pancreatic islets, i.e. insulitis. Injection of 3-O-methyl glucose, nicotinamide or ALS in low-dose STZ-treated mice retarded development of hyperglycaemia during the course of treatment. However, diabetes developed shortly after treatment ceased (484), demonstrating the requirement of a cell-mediated response elicited by direct STZ-mediated β-cell injury.

Infiltrating cells comprise macrophages as well as T and B cells. However, despite the detection of autoantibodies following subdiabetogenic doses of STZ (485), mice exhibiting B lymphocyte deficiency responded to multiple low-dose STZ-diabetogenesis. This data does not support a primary role for humoral immunity in this animal model (486). Macrophages and neutrophils are probably the earliest infiltrating cells. Macrophages develop spontaneous cytotoxicity against islet cells *in vitro* (487), and the cytokines IL-1β and TNF secreted by activated macrophages are extremely cytotoxic to cultured islet cells, particularly in combination with IFN-γ (488). In addition, high concentrations of IFN-γ and TNF have been shown to act synergistically to induce MHC class II antigens on islets *in vitro* (489). The expression of class II antigens on islet β-cells may play a role in the pathogenesis of IDDM (490, 105). Anti-inflammatory steroids were found to be strongly immunoprotective in multiple low-dose STZ mice diabetogenesis (491).

Kolb (492), in a review of the literature describing the effects of immunomodulatory compounds on low-dose STZ-diabetes in the mouse, reported that nearly all compounds suppressing T lymphocyte or macrophage function, including antibodies against MHC class II I-A and I-E, Thy-1, Lyt-2, whole body UV irradiation, anti-inflammatory steroids, agents inhibiting serotonin-enhanced vascular

permeability, and silica, partially suppress development of diabetes. Only administration of CsA failed to suppress diabetes (493). On the contrary, CsA enhanced the development of low-dose STZ-diabetes, probably as a result of the combined toxicity of CsA and STZ on mice islet β-cells (494).

The contribution of the immune system to the destruction of the β-cell mass is clearly demonstrated in this animal model. However, multiple low-dose STZ-diabetes is not truly autoimmune in nature, and only one report has claimed to successfully transfer overt diabetes to naive recipients using splenocytes from low-dose STZ-diabetic mice (495). In this study, recipients were only monitored over a short period of time and the level of hyperglycaemia reported was modest. More typical is the report of Kim and Steinberg (496) who failed to adoptively transfer diabetes into normal male mice receiving splenocytes from low-dose STZ-diabetic syngeneic donors.

Further evidence demonstrating lack of autoimmunity in these mice came from islet transplantation studies. Transplantation of syngeneic islets into diabetic recipients in numbers sufficient to reverse hyperglycaemia did not result in autoimmune destruction of the islet graft at a time when insulitis was present in the recipients native pancreas (497). If cell-mediated immunity is involved in low-dose STZ-induction of diabetes, an explanation as to why islets were not rejected involves their modification by STZ, implying that β-cells must be altered to render them antigenically distinct from normal β-cells. Indeed, recipients of an intrasplenic syngeneic islet graft treated following transplantation with STZ became hyperglycaemic (498), confirming that β-cells had to be altered for an immune reaction to ensue.

In summary, the multiple low-dose STZ-diabetic mouse represents a useful model in which to study the consequences of insulitis and IDDM. However, the diabetic

condition is primarily produced by an environmental toxin, and not by a loss of tolerance to β-cell specific antigens in a genetically susceptible individual. In this respect, pathogenesis in this animal model is distinct from autoimmune diabetes of humans, BB rats and NOD mice.

1.3.4. **CONCLUSIONS**

Studies involving animal models of diabetes have greatly contributed to the elucidation of the sequence of events in the pathogenesis of IDDM. The resemblance of these models, particularly the BB rat and NOD mouse, to human autoimmune IDDM allows us to tentatively extrapolate this information and use it in the unravelling of events leading to the onset of the human diabetic condition. However, initiation factor(s) resulting in the onset of this disease have still to be determined in both animal models and human IDDM. Until then, primary prevention of human IDDM will remain elusive and, in addition, the very real problem of accurately predicting individuals susceptible to diabetes still exists. For these reasons, major efforts concentrating on improving methods of insulin treatment and delivery appear more worthwhile at the present time.

1.4. **IMPROVED METHODS OF INSULIN DELIVERY**

1.4.1. INSULIN THERAPY

The introduction of insulin remains the single most important milestone in the history of diabetes, and over the seven decades since subcutaneous insulin injections were first given, no other therapy has displaced insulin as the principal treatment for diabetes. Insulin treatment reverses the diabetic state and restores an anabolic

condition where fuel is stored and released roughly in keeping with need. To achieve this balance, insulin must be provided to approximate the variations occurring in non-diabetic individuals, with increases at meal-times and subsequent decreases to appropriate basal amounts in the post-absorptive periods of the day.

In the therapeutic sense, short-acting insulins are ideally suited to the requirements of primary meals (breakfast and dinner) , while admixed intermediate-acting insulins can accommodate the secondary meals (lunch and supper) as well as provide the extended basal requirements overnight (499,500). All currently available intermediate-acting insulins have a duration of less than 24 hours (501) and therefore if the patient releases no endogenous insulin, twice-daily injection is required (502).

It has been noted that the actual metabolic control achieved with daily insulin injections and monthly interventions for dosage adjustments is inferior to that occurring naturally in the non-diabetic population, and close-to-normal blood glucose values were not achieved. The recently released findings of the Diabetes Control and Complications Trial (503) clearly indicate that intensively managed IDDM patients maintain lower blood glucose than fairly well controlled conventionally-treated patients. More importantly, intensification of insulin therapy led to a substantial reduction in the development or progression of retinopathy, nephropathy and neuropathy, as well as a likely reduction in macrovascular complications. However, it should be noted that the long-term complications of IDDM are reduced rather than prevented. In addition, a three-fold increase in risk of severe hypoglycaemia and modest excess weight gain are observed in intensively managed patients (504), and a considerable increase in patient effort will be required to reproduce the results of the Diabetes Control and Complications Trial in practice, and at a substantial cost.

Entry of insulin into the body primarily determines insulin levels and action and constitutes the principal problem with current therapy since the disappearance of insulin from the bloodstream is fast (half-life of approximately 3-5 minutes in non-diabetic subjects). A discussion of these difficulties is therefore helpful in understanding the need for alternative approaches to insulin delivery.

1.4.1.1. POOR COMPLIANCE

Poor compliance is a general problem of injection therapy which has recently been exacerbated by the need to inject insulin more frequently with optimised regimens than with previous treatments employing once or twice-daily injections. The introduction of the insulin pen containing a pre-filled cartridge of short-acting, intermediate-acting or pre-mixed insulin, has greatly improved compliance since it is convenient, portable, and less painful on injection compared with syringes and needles (505).

1.4.1.2. ADVERSE INSULIN PHARMACOKINETICS

The non-physiological delivery of subcutaneous insulin is thought to lead to hyperinsulinaemia in many patients, probably due to under-insulinisation of the liver. In non-diabetic individuals, portal insulin is at least twice as high as the systemic insulin concentration (506). The post-receptor resistance to insulin which is present in IDDM patients (507), and the lack of pulsatility of exogenous insulin delivery in contrast to the approximately 10 minute episodic secretion of insulin in normal man, may also contribute to hyperinsulinaemia.

In addition, the absorption rate of both short and delayed-acting insulin is highly unsatisfactory. After subcutaneous injection of short-acting insulin into the diabetic patient, the plasma free insulin concentration rises slowly to a broad plateau level approximately 40-120 minutes after injection, after which the insulin gradually falls to baseline levels some 6 hours after injection. This slow absorption may reflect the time taken for hexameric insulin to dissociate into dimers and monomers after injection, the forms in which insulin is absorbed into capillaries (508). In contrast, after a mixed meal in non-diabetic individuals, plasma insulin reaches a peak approximately 45 minutes before returning to basal concentrations some 4 hours later. IDDM patients would therefore be insulin deficient in the first minutes after a meal if insulin was injected at the start of eating. Thus insulin is now administered approximately 30 minutes prior to a meal in an attempt to match peak plasma free insulin and glucose concentrations (509,510). The slow fall to baseline of plasma free insulin concentrations after injection of short-acting insulin is also problematic and may result in marked overinsulinisation between meals which carries a consequent risk of hypoglycaemia.

Delayed-action insulin formulations fail to provide a constant basal supply of insulin, have different absorbance rates, and plasma free insulin plateaus are observed 3-6 hours after injection (511). Insulin levels decline approximately 10 hours after injection, thus delayed-action insulin injected in the early evening does not last through the night, resulting in insulin deficiency and hyperglycaemia in the few hours before breakfast. Although increasing the dose of these insulins increases their duration of action, peak insulin levels are also increased and occur unpredictably (512). Erratic hypoglycaemia may therefore occur during the night.

1.4.1.3. VARIABILITY OF INSULIN ABSORPTION

Species of insulin is of some importance in influencing its absorption, and both short-and delayed-acting human insulins are absorbed slightly more quickly (and are therefore of shorter duration) than equivalent porcine preparations (513,514).

For maximum comfort and effect, insulin should be injected into the adipose layer under the skin. Occasionally patients unintentionally inject insulin intramuscularly (i.m.), particularly when injecting perpendicularly rather than obliquely into a lifted skin-fold. In the thigh, absorption of injected insulin from muscle is approximately 50% higher than from the subcutaneous site (515). Infections at the injection site are rare.

The anatomical site of insulin injection also influences absorption rate, and short-acting insulin is most rapidly absorbed from the abdomen, with intermediate rates from the arm and a slower rate from the gluteal region (516,517). For consistency purposes, injections should be rotated within a given area. This also prevents lipohypertrophy, i.e. deposition of fatty lumps resulting from repeated injections of insulin in the same place, which slows insulin absorption (518). Insulin absorption is augmented by heat (519), massage (520) and exercise (521).

1.4.1.4. ALTERNATIVE ROUTES FOR INSULIN DELIVERY

The subcutaneous route for insulin delivery has proved its efficacy and practicality for self-management by diabetic patients over a long period. However, as discussed above, several disadvantages exist, thus an alternative route for administration of insulin, which is less aggressive and more effective, is desirable.

1.4.1.4.1. The intranasal route

The first attempts to administer drugs into the body through the nasal mucosa were made in the 1920's (522). The more recent discovery that surface-active agents such as bile salts and glycocholate enhance insulin absorption through mucosa (523) has led to renewed interest in the intra-nasal route of insulin administration. Intranasal insulin, when given as a spray, has been shown to lower fasting and postprandial blood glucose in normal subjects and IDDM patients (524). Serum levels of insulin rise within 10-15 minutes. The half-life of insulin in the plasma is short, and the hypoglycaemic effect only lasts for approximately 30-75 minutes. The insulin level observed is linearly dose dependent, but unfortunately, bioavailability is low and only 10-30% of the insulin dose is actually absorbed. Possible adverse long-term effects on the nasal mucosa in the presence of a surfactant that is irritating, if not destructive, may limit the use of the nasal route for insulin administration. Nasal insulin sprays might therefore only be used as an adjunct to therapy in diabetic patients treated with subcutaneously administered insulin, and in IDDM patients on long-acting insulins.

1.4.1.4.2. The gastrointestinal route

Although administration of insulin via the gastrointestinal route would be comfortable and convenient, the enzymatic digestion of all ingested peptides is a critical factor which has yet to be overcome.

The absorption of insulin through the mucosa of the mouth is extremely low. However, when placed directly in the jejunum of animals, insulin is effectively absorbed through the mucosa (525). Insulin has also been protected from degradation by entrapment in positively charged liposome vesicles (526,527)

although the bioavailability of insulin was only 1-4%, variable and rarely predictable (528). Attempts have also been made to delay the absorption of orally administered insulin until in the colon by coating the hormone with polymers that are only susceptible to digestion by the colonic microbial flora (529).

Another mode of administration of exogenous insulin which prolongs its action and thereby improves the efficacy of glycaemic control is the use of sustained-release implants for insulin delivery.

1.4.2. SUSTAINED RELEASE INSULIN IMPLANTS

A common aspect of all sustained release insulin implants is the presence of a barrier to delay contact between body fluid and the insulin contained within the implant. This barrier can take the form of an erodible excipient which dissolves gradually *in vivo*, thus releasing the entrapped insulin at a constant basal rate.

One of the first attempts to use sustained release of insulin in the treatment of diabetes was performed by Davis (530), who subcutaneously implanted polyacrylamide admixed with insulin into alloxan-induced diabetic rats. The response of the implants was found to be influenced by implant porosity, which was dependent upon the polyacrylamide concentration and the amount of insulin present. Animals bearing 10 mg insulin implants of 40% PAA showed no glycosuria and grew at virtually the same rate as control rats, until removal 21 days after implantation led to recurrence of diabetes. More recent advances in this approach of insulin treatment have been reported by Brown and co-workers, who developed a biocompatible system that achieved a continuous release of biologically active insulin from small polymer matrices (531). Powdered insulin particles were incorporated into an ethylene-vinyl

acetate copolymer matrix which has previously been shown to be biocompatible *in vivo* by this group (532). The presence of particulate insulin resulted in a matrix of tortuous channels and constricted pores through which insulin could be released upon influx of aqueous media. The diffusion of the dissolved insulin through this tortuous network was driven by the large concentration of insulin within the matrix. The insulin release kinetics could be enhanced by increasing the powder particle size (resulting in larger pores), loading (resulting in a greater number of pores), solubility of insulin, and porosity of the matrix. This group further investigated the effect of ethylene-vinyl acetate copolymer matrices on STZ-diabetes in the rat (533). The 60 mm^3 implants designed to release insulin at near constant rates normalised plasma glucose concentrations and eliminated glycosuria. These parameters were controlled for up to 105 days by a single implant, and glycated haemoglobin (HbA_1) values measured 90 days after implantation were reduced compared with values for the diabetic controls, and were similar to HbA_1 values observed in normal rats. The average weight gain of the implant-treated rats was also similar to that of controls, whereas diabetic controls failed to thrive and developed cataracts one month after the induction of diabetes. As anticipated, plasma glucose levels began to increase at approximately the same time as the release rates of insulin from the implants decreased *in vitro*.

More recently, Wang (534) investigated the use of naturally occurring materials to form the matrix of sustained release insulin implants (SRII). Initially, the insulin release from a cholesterol matrix implant containing differing amounts of insulin was evaluated in STZ-diabetic rats, and its effect on hyperglycaemia noted. At 3% insulin, two pieces of quarter-length implants (approximately 50 mgs each) caused a slight short-term decrease in blood glucose levels, although recipients were still hyperglycaemic. Doubling the insulin content of SRII to 6% normalised and sustained

blood glucose levels for approximately 10 days. This normalisation was increased to 24 days using 8-10% insulin in the matrix composition. Three successive subcutaneous insertions of 80 mg pellets containing 10% insulin maintained normoglycaemia for up to 10 weeks. Increasing the insulin content of the implants to 50% resulted in severe hypoglycaemia when implanted into STZ-diabetic rats. Additionally, as cholesterol is naturally occurring and found in all animal tissues, no inflammatory fibrotic response was observed against the implant, thus demonstrating the suitability of cholesterol as a biocompatible material. However, it should be noted that the cholesterol implant did not dissipate in the recipient, and was still present after one year. This is an obvious disadvantage when considering the insulin-containing cholesterol implant as a potential treatment for the diabetic patient.

Subsequently, Wang replaced the cholesterol excipient with palmitic acid (535), which gradually erodes in the rat with a half life of approximately 3 months. These SRII were used to investigate their reproducibility in insulin delivery and ability to afford sustained reduction of hyperglycaemia in STZ-diabetic rats. A one-eighth size piece of pellet containing either 10%, 15%, 20% or 30% insulin by weight was implanted subcutaneously into the recipient and blood glucose levels measured daily. Implants with a 10% or 15% insulin content had an unpredictable activity, whereas all of the implants with a 30% insulin content delivered too high a dose of insulin, causing fatal hypoglycaemia. The delivery and onset of action of a SRII containing 20% insulin content were both highly reproducible. However, variations in the service life of these essentially identical implants were larger than anticipated. Overnight fasting studies showed that diabetic rats implanted with a one-eighth size piece of implant containing 20% insulin could tolerate the induced hyperglycaemia without experiencing convulsions.

Although these alternative methods of insulin administration may be promising, the classic subcutaneous route of administration is not likely to be substituted in the near future. However, a serious deficiency in all these current insulin treatments is the inability to automatically adjust the rate of insulin delivery according to the prevailing blood glucose concentration. In the non-diabetic individual, the islet cells of the endocrine pancreas continuously monitor blood levels of several metabolites and secrete appropriate amounts of insulin to achieve a delicate metabolic balance during periods of feeding and fasting. In comparison, exogenous insulin therapy is considerably less sophisticated and through the use of technology, insulin delivery requires substantial improvement if imitation of the intricate insulin profiles of non-diabetic subjects is the therapeutic goal for treatment of diabetic patients.

1.4.3. GLUCOSE SENSORS AND CLOSED-LOOP INSULIN DELIVERY

In 1974, a novel insulin delivery system was described that regulated the rate of insulin delivery in response to measured blood glucose levels (536,537). This device quickly attained and maintained normoglycaemia as a direct consequence of its closed-loop nature and inherent characteristics of negative feedback control (538). In addition, blood was sampled every minute, thereby allowing insulin delivery to be appropriately adjusted on a continual basis. The low basal infusion of insulin could be combined with appropriately and precisely added insulin at meal-times. This system clearly promised to reverse diabetes and to normalise blood glucose control with a precision far greater than that achieved using the most intensive of conventional insulin treatments (CIT). However, because the venous route is used for blood sampling and insulin delivery, and the apparatus is relatively large and complex, this system is unsuitable for studies of more than a day or so in duration. Efforts have therefore turned to miniaturisation of the device and to the development of a blood

glucose sensor which can be implanted in the body over extended periods of time at a site not likely to lead to septicaemia and thrombosis, both of which are risks of intravascular sensing.

1.4.3.1. TYPES OF GLUCOSE SENSOR

Most glucose sensors are enzyme electrodes which use the immobilised enzyme, glucose oxidase, layered on the surface of the electrode to catalyse the oxidation of glucose to gluconic acid and hydrogen peroxide. Glucose levels can be measured by monitoring either the consumption of oxygen or by production of hydrogen peroxide or hydrogen ions. Amperometric electrodes detect a current change at a set voltage whereas hydrogen ions may be detected at one of several types of pH electrode. These are potentiometric (voltage-measuring) sensors in which there is an alogarithmic relationship between analyte (glucose) activity and voltage change. Technical problems of these sensors include significant drift, necessitating frequent recalibration, difficulties in sealing electrical connections in a liquid environment and reduced pH due to the buffering capacity of biological fluids.

One variation of the amperometric biosensor is the mediated electrode in which a small molecular weight redox couple, i.e. the oxidised and reduced forms of a substance such as ferrocene ferricinium ion, shuttles electrons from glucose oxidase to the base electrode (539,540). These sensors are relatively oxygen insensitive because molecular oxygen is not the final electron acceptor. The technology has been successfully commercialised as the Exactech Pen (MediSense, Cambridge, MA) for *in vitro* glucose analysis (541).

Fibre optic-based glucose sensors have not reached the stage of *in vitro* testing but are attractive in principle because the sensing element is miniature and the fibres are cheap to manufacture, electrically isolated from the patient, and do not need a reference electrode. Glucose can be measured by immobilisation of glucose oxidase and the resultant pH change can be detected by fluorescence quenching (542) or colour change of a dye (543). Oxygen consumption in the glucose oxidase reaction can be measured with an oxygen sensitive fluorescent dye (544) and hydrogen peroxide by the chemiluminescence generated from the reaction of hydrogen peroxide with an oxalate derivative and surface enhancer (545).

Long-term glucose sensing in the vascular compartment has generally been avoided, mainly due to the clinically unacceptable risks of septicaemia, thrombosis and embolism. However, Armour et al (546) have successfully operated an oxygen-detecting type of glucose sensor in the vasculature of dogs for several weeks. The subcutaneous tissue is a more favourable site for the implantation of a glucose sensor as it is easily accessible and safe, although some infection or inflammation may be observed. Furthermore, studies suggest that the glucose concentration in the subcutaneous interstitial fluid is almost identical to the plasma glucose concentration (547). The time lag between changes in blood glucose levels during a glucose load and the generation of a current by the sensor was usually less than 15 minutes when implanted in the subcutaneous tissue of rats (548), dogs (547,549), and humans (550). This slight delay in subcutaneous changes after rapid increments or decrements in blood glucose concentration would not negate the clinical usefulness of this implantation site.

Bindra et al (551) have developed a miniaturised glucose sensor (outer diameter, 0.45 mm) which can be readily implanted in the subcutaneous tissue and replaced

every 3 or 4 days. This sensor has been extensively evaluated in rats, where it has been shown to work for up to 10 days (548,552) and dogs (553,554). In dogs, oscillations in current occur after some days, preceding a decline in sensitivity of the sensor (549). This failure is accompanied by swelling and exudation at the implantation site, and since the exudate fluid is of a low glucose concentration it has been proposed that inflammatory cells may metabolise glucose and contribute to sensor impairment. The current generated by the sensor was transformed into an estimation of the concentration of glucose by using the sensitivity coefficient and an extrapolated background current in the absence of glucose. However, this calibration was performed on the basis of the retrospective analysis of the changes in blood glucose and the current generated by the sensor, and for clinical application of the system, an on-line estimation of glucose is necessary. A method for on-line calibration using a portable, battery operated, monitor with software which immediately transforms the measured current into an estimate of glucose concentration has recently been reported (555).

Although few studies have looked at the performance of implanted glucose sensors over several days in human subjects, all enzyme electrodes required replacement after periods ranging from several hours to days (540,556). After implantation, output initially declines but stabilises several minutes to hours later.

Microdialysis which involves sampling of blood or interstitial fluid and analysis by an *ex vivo* glucose sensor (557,558) may reduce problems of bioincompatibility. Hollow fibres of dialysis membrane are implanted, often subcutaneously, perfused at a low flow rate with isotonic fluid and the perfusate pumped to a flow-through cell outside the body that incorporates a glucose sensor. Such devices function well over one day, but long-term biocompatibility at the fibre/tissue interface and the

practicability of the device need to be determined. Bolinder et al (559) employed microdialysis in a short-term study of diabetic patients, and reported that absolute glucose concentration could be measured directly. More recently, this group (560) reported that microdialysis of adipose tissue can be used for continuous long-term monitoring of glucose concentrations in diabetic patients during ordinary daily life. Most patients had a reproducible daily glucose profile, and when patients' insulin therapy was adjusted on the basis of ambulatory glucose monitoring, HbA_{1c} values decreased by almost 2%, and this decrease lasted for at least 9 months. In this study, tissue dialysate glucose measurements and data analysis were again retrospective, although future improvements will include development of on-line glucose analysis.

In spite of considerable progress in technology, long-term *in vivo* glucose sensing has not progressed to routine clinical practice. This is largely due to a number of problems with glucose sensors, including diminished sensitivity *in vivo*, and unpredictable drift and bioinstability at the output (561). Sensors calibrated initially *in vitro* in buffered glucose solutions may suffer a reduction in sensitivity of approximately 10-80% on implantation (539,540), necessitating some *in vivo* calibration procedure. This impairment of response is usually ascribed to inhibitory substances in the tissues that interact with the enzyme, base electrode or reaction products, coating of the sensor with protein or cellular material from the biological matrix. Covering membranes have not resolved these problems, as they may also be overgrown or ingrown by body tissue, and eventually simply rejected as foreign. These devices therefore appear to be more applicable for short-term rather than long-term monitoring of *in vivo* glucose concentrations.

In vivo glucose sensing and its inherent problems may soon be superseded by non-invasive glucose monitoring, which would be highly acceptable to diabetic

patients. Near-infrared (NIR) spectroscopy is the most promising approach since, in the region of 700-1000 nm, radiation passes through several centimetres of tissue. Specific glucose detection by analysis of NIR absorption peaks has proved difficult because of the large band due to water, the variable scattering of light in tissues which alters the optical path length unpredictably, and the overlapping absorption of many metabolites in this region. However, several groups are now reporting good correlation between blood glucose and NIR transmission (562,563). It should be noted that NIR spectroscopy has not yet been miniaturised to a form that could be used as a wearable *in vivo* monitor, and the cost of such a device is likely to be high. Although NIR spectroscopy resolves the problem of biocompatibility, calibration of the system requires further study.

1.4.4. CONCLUSION

Substantial evidence implicates the involvement of abnormal blood glucose in the development of micro- and macroangiopathic complications of IDDM. Normal or near-normal glucose homeostasis may slow or even halt the progression of these complications both in experimental animals and in humans, although it is not known how early in the progression of the disease normal glucose control must be restored to have an effect. In IDDM, blood glucose control is normally maintained by subcutaneous insulin administration. However, CIT cannot replace the complex physiological balance of endogenous insulin released from normal islets which regulates glucose homeostasis. Even when insulin administration is intensive and carefully controlled by multiple daily injections, blood glucose concentrations in patients with diabetes can swing widely outside the normal range. Alternative routes for insulin administration, including nasal and gastrointestinal routes, are also problematic and are unlikely to replace the conventional subcutaneous route of insulin

administration at this time. Sustained release insulin implants release a constant basal dose of insulin upon implantation into animal models, thereby removing the need for daily insulin injections. The implants can achieve near-normal blood glucose levels over a period of several months. The major problem with all the insulin therapies discussed so far is their inability to respond to changes in blood glucose concentration, thereby leading to poor metabolic control. Glucose sensors can measure blood glucose every minute and using this information, insulin delivery could be adjusted on a minute-to-minute basis, thereby attaining precise blood glucose control compared with the aforementioned non-responding forms of diabetes management. However, these closed-loop systems do not function *in vivo* long-term mainly due to the unreliability and bioincompatibility of glucose sensors . If suitable covering membranes for the sensor are discovered that exclude interfering substances or control coating or encapsulation with proteins and cells, the excellent performance *in vitro* may be matched *in vivo*.

An alternative approach to insulin therapy as treatment for IDDM patients is to replace the destroyed pancreatic islets, thereby restoring normal metabolic control. This can be achieved by transplantion of vascularised whole or segmental pancreas or isolated pancreatic islets.

1.5. **PANCREAS TRANSPLANTATION**

The first human transplant of a vascularised pancreas was reported in 1967 by Kelly and co-workers (564) who showed that the technique was feasible and could restore blood glucose levels to normal in patients with type I diabetes. After this pioneering attempt, there was a proliferation of centres offering the procedure, and in the past

15 years more than 4000 such operations have been done, nearly 70% of them since 1987.

An important consideration for diabetic patients receiving a pancreas transplant is that while gaining independence from administration of daily insulin, they become dependent upon continuous immunosuppressive medication, even in HLA-identical donor pancreas transplants. Long-term immunosuppression can have multiple toxic effects, including increased risks of infections and malignant disease. Side effects of the triple immunosuppression regimen most commonly used to prevent graft rejection include azathioprine myelosuppression, cyclosporin nephrotoxicity, and steroid induced gastrointestinal bleeding and perforation. Cyclosporin and steroids also increase blood pressure and alter the lipid profile, thereby possibly neutralising some of the cardiovascular benefits of achieving normoglycaemia. A non-uraemic diabetic patient whose hyperglycaemia is manageable by CIT would therefore be unwise to ignore these risks simply in the hope of avoiding dependence on insulin injections. However, if the secondary complications of diabetes were prevented, arrested or even reversed, then the risks involved in pancreas transplantation may seem more acceptable.

Three years after a successful pancreas transplant, patients showed no differences in the severity of their retinopathy compared with those patients having had an unsuccessful operation (565). Studies on diabetic neuropathy are more encouraging, and sensory, motor and autonomic indices have shown benefits from successful pancreas transplants in contrast to patients with failed grafts or receiving no transplant (566). Renal function has been reported to deteriorate more rapidly after pancreas transplantation (567), probably due to cyclosporin renal toxicity (568), and seems to stabilise after the first year or so. Measurements of glomerular structural

alterations in the native kidneys five years after a pancreas transplantation did not show a clear benefit in the transplanted groups, compared with those in non-transplanted patients with controlled IDDM (569). Moreover, a significant decline was observed during the study period in transplanted patients, whereas glomerular filtration rate was virtually unchanged in the comparison group. The inability of pancreas transplantation to show any amelioration of complications in the diabetic patient is probably attributable to the fact that transplantation is performed at a late stage of the disease, when complications are apparently irreversible.

There is growing evidence that kidney transplantation is the preferred treatment in uraemic diabetic patients rather than long-term dialysis. However, recurrence of diabetic nephropathy has been observed in the transplanted kidney even as early as 2 years after transplantation in diabetic patients (570). For these reasons, combined kidney and pancreas transplantation would be advantageous for the diabetic uraemic patient, removing dependence on both dialysis and insulin therapy. Since renal transplant patients are treated with immunosuppressive drugs, further immunosuppression is unnecessary. The transplanted kidney seems to have a protective role with regard to the transplanted pancreas, as shown by the International Pancreas Transplant Registry (571), which reports a one year survival rate of 49% when pancreas was transplanted alone (n = 92), 45% for patients with a previous kidney transplant (n = 145), and 75% when pancreas and kidney were transplanted simultaneously (n = 1360). The improved pancreas survival when combined with a kidney transplant may be due to earlier diagnosis and treatment of rejection. However, patients with combined transplantation have three times more acute rejection episodes than those with kidney transplants alone (572,573), possibly because pancreas grafts are more immunogenic than kidney grafts.

Management of the exocrine function of the transplanted pancreas remains a major problem. Among the different methods proposed, three approaches are commonly employed : polymer injection (574), the pancreaticoduodenal technique (575) and urinary diversion (576). Data from the International Pancreas Transplant Registry (571) show a significantly higher survival for bladder drained grafts, although bladder drainage carries the risks of bladder leakage, bleeding, abcesses, haematuria, urethral stricture, stone formation and bicarbonate loss.

Thus, considerable morbidity and substantial mortality is still associated with combined pancreas and kidney transplantation. The 1 year patient survival is 85% for the combined operation compared with 96% for living related kidney transplant alone and 93% for cadaveric kidney alone (571). The age of the patient is an important variable : patients under 45 years of age have approximately the same 1 and 2 year survival rate with combined transplant as with kidney transplant alone, whereas patients older than 45 years have only 33% survival rate at 1 year after the combined operation (577).

The impact of combined transplantation on diabetic complications is difficult to determine as formal comparison with solo kidney transplantation has not been made. Landgraf et al (578) reported an improvement in peripheral neuropathy after combined transplantation, but similar findings were observed with kidney transplantation alone, which were not improved by the subsequent addition of a pancreas graft (579). Conflicting results have been obtained concerning diabetic retinopathy : improvements have been reported after the combined operation (578), whilst others report comparable visual acuity and retinal appearance in patients who have had a successful or a failed pancreas transplant (565). Patients receiving a pancreas transplant a few years after successful kidney transplantation reportedly

prevents the development or progression of the earliest of diabetic glomerulopathy compared with patients who received no subsequent pancreas graft (580). However, many diabetic patients receiving kidney transplants alone develop few or no lesions of diabetic nephropathy in the first 6-14 years after transplantation (581).

Thus, successful combined kidney and pancreas transplantation can free the diabetic patient from daily insulin injections and dietary restrictions. Good metabolic control is also achieved, with restoration of normal fasting plasma glucose levels, 24-hour plasma glucose profiles, plasma glucose changes during oral and i.v. glucose tolerance test (IVGTT) (582), and normalisation of HbA_{1c} and intermediary metabolite levels (583). However, these improvements in patients quality of life must be weighed against the the complications of surgery, side-effects of life-long immunosuppression, considerably increased morbidity, very high mortality among older patients and increased cost. Furthermore, the benefits of pancreas transplantation, alone or combined with a kidney transplant, on long-term diabetic complications are disappointing, probably because these operations are currently performed at a late stage in the disease. A vascularised pancreas graft is unlikely to be considered if early transplantation means transplantation into a patient who is young, fit and simply inconvenienced by daily insulin injections. At this stage of IDDM, the associated peri-operative morbidity of pancreas transplantation and the immunosuppression required could not be justified.

Isolated pancreatic islet transplantation has a number of actual and theoretical advantages over vascularised pancreas grafts : transplantation of a small volume of pure islet tissue is a relatively simple and minor procedure, with no exocrine complications. In addition, pretreatment might reduce islet immunogenicity, and storage by cryopreservation would allow optimal matching of donor and recipient, as

well as transplantation of islets from more than one donor into single recipients. Foetal pancreas transplantation presents similar advantages, and also provides a graft in which β-cell replication can occur. However, the use of foetal tissue is associated with major ethical difficulties.

1.6. ISLET TRANSPLANTATION IN ANIMAL MODELS OF IDDM

Pancreatic islets have been isolated for many years by a technique described by Ballinger and Lacy (584) in which the distended pancreas is chopped into small pieces, digested with collagenase, and the released islets separated on a density gradient. Although the preparation of islets is pure, the yield is relatively poor. An alternative technique for isolating rat pancreatic islets has more recently been described : the pancreas is distended with collagenase injected via the pancreatic duct and digested statically at 37°C. Reproducibly high numbers of intact islets are obtained (585,586) which maintain biphasic insulin secreting properties *in vitro* in response to a glucose stimulus indicating that the islets remain viable after the isolation procedure.

1.6.1. STZ-DIABETIC MODELS OF IDDM

Early experimental work (584) looked at the transplantation of isolated pancreatic islets into the chemically-induced STZ-diabetic rat. A small number (400-600) of syngeneic islets implanted i.p. or i.m. resulted in a significant reduction of hyperglycaemia, polyuria and glycosuria and a normalisation of weight gain. Excision of the transplanted islets from the muscle resulted in a return to the fully diabetic state, and upon histological examination islets were found to be intact but with β-cell degranulation, probably due to the great demand for insulin in these animals.

Exogenous insulin given for a period of time after transplantation may lessen the extreme demand upon the islet graft to secrete insulin and afford the transplanted islets some degree of protection.

Under normal circumstances, insulin from native pancreatic β-cells is secreted directly into the portal venous system. Kemp et al (587) therefore suggested that the intraportal site may provide a more physiological environment for the transplanted islets and allow a more effective utilization of secreted hormones than islets implanted i.p. or subcutaneously. 400-600 islets injected intraportally into the STZ-diabetic rat also achieved normoglycaemia, normal urine volumes and abolition of glycosuria, and these parameters were maintained for up to two months after transplantation. Islets implanted i.p. or i.m. gave similar results to those reported previously (584). This data suggests that the site of implantation is an important consideration in islet transplantation studies. Ideally the site would be safe and permit maximum survival and function of transplanted islets and maximum effectiveness of secreted hormones.

Many studies followed these initial islet transplantation experiments. Reckard et al (588) reported that an i.p. injection of 600-1200 syngeneic islets into STZ-diabetic rats rendered the recipient normoglycaemic for as long as 11 months. However, insulin response to an IVGTT was sluggish and 25% of test animals returned to a state of hyperglycaemia 5-6 months after islet transplantation. Similar numbers of allogeneic islets initially fared as well as the syngeneic grafts but recipients returned to a state of hyperglycaemia within 12 days of transplantation. In contrast with syngeneic islets, transplanted allogeneic islets were vulnerable to rejection and could evoke an immune state characterised by subsequent accelerated rejection of donor strain skin grafts.

Islet number is another important factor for successful transplantation. The neonatal pancreas 2.5-4.5 days before birth reportedly has the highest insulin content, the largest islet volume percentage, and the lowest exocrine enzyme concentration during the foetal or postnatal period (589,590), thus indicating its suitability for transplantion studies. Islets isolated from 25-35 neonatal rat pancreases were transplanted i.p. to chemically-induced alloxan-diabetic rats (591). Diabetes was reversed within several days following the islet isograft and normoglycaemia persisted for more than 5 months. Islets injected into the peritoneal cavity were associated with the liver, spleen, pancreas and abdominal wall and vascularised grafts consisted of heavily granulated β-cells. In alloxan-induced diabetic rats allografted with similar numbers of dissociated neonatal pancreases, 46% showed a transitory recovery from their diabetes lasting 3-13 days, before returning to a pretransplant diabetic state. Again, failure of the transplant was attributed to rejection, and upon removal the graft showed evidence of degenerating β-cells and lymphoid infiltration indicative of the rejection process.

The criteria used to assess the effectiveness of islet transplantation in reversing the diabetic state have so far involved rather gross indices of severe insulin deficiency, i.e. fasting blood glucose level, degree of polyuria, glycosuria and weight loss. Pipeleers-Marichal et al (592) determined the extent to which islet transplantation could normalise the severely insulin deficient rat. 350-1000 isolated islets injected intraportally into STZ-diabetic Lewis rats resulted in a normalised fasting plasma glucose and insulin levels within 24 hours. Polyuria, polydipsia and hyperglucagonaemia disappeared more gradually over a 2-12 week period, i.e. the time required for normalisation varied with the severity of the diabetic recipient and the number of islets transplanted. Long term transplanted islets were shown to

establish direct contact with surrounding hepatocytes and demonstrated well granulated α-, β- and δ- cells.

Gray and Watkins (593) also confirmed these findings and suggested that the portal venous site was optimal for reception of transplanted pancreatic islets. Again, allogeneic islets, even when transplanted across a weak histocompatible barrier, were shown not to survive for long periods of time unless recipients were immunosuppressed. Recipients of allogeneic islets after a period of immunosuppression with ALS became permanently tolerant to the transplanted islets and to subsequent donor specific skin grafts.

The spleen also possesses a portal venous drainage and, as with the liver, is a highly vascular organ, ideal for providing an environment for the successful nutrition of transplanted islet tissue. However, unlike the liver, the spleen is expendible and therefore allows graft retrieval should irreversible host organ damage ensue. Finch et al (594) injected adult rat islets into the splenic pulp of syngeneic and allogeneic STZ-diabetic recipients. Normal serum glucose levels and 24 hour urine volumes were restored in a mean of 3.3 days. Syngeneic recipients remained normoglycaemic for 6 months and splenectomy performed 110-178 days after transplantation resulted in a prompt return to the diabetic state. In contrast, allogeneic grafts were rejected in a mean of 5.2 days unless recipients were treated with a short course of ALS, which resulted in normoglycaemia for at least 4 weeks. The results using the spleen as the implantation site were not significantly different from those recorded in comparable groups receiving intraportal allogeneic islets. In contrast, Reckard et al (595) found that in order to achieve moderately good control of glucose homeostasis in the STZ-diabetic rat, twice as many islets were required

when the spleen was used as the implantation site compared with successful intraportal transplantation.

A peritoneal-omental pouch constructed by encasing the omentum in a pouch formed from a strip of the hosts parietal peritoneum was successfully used as an implantation site for an islet isograft in STZ-diabetic rats (596). Islets placed in the pouch were in close apposition to the omental blood supply and normoglycaemia was subsequently achieved in diabetic recipients. The removal of the islet bearing peritoneal-omental pouch 6 weeks after transplantation resulted in a prompt return to the diabetic state in all recipients, and histologically, islet aggregates (surrounded by adipose tissue) were well vascularized and the β-cells had a normal degree of granulation.

Brown et al (597) used another transplantation site, beneath the kidney capsule, for the implantion of 2-3 foetal syngeneic pancreases in STZ-diabetic Lewis rats. The experimental diabetes was partially reversed provided that insulin was given for several days following transplantation to prevent overexhaustion of the islet β-cells and allow maturation of the graft. However, foetal allogeneic pancreases implanted under the renal capsule were rapidly rejected, indicating that foetal tissue may not be less immunogenic than adult pancreatic islets (598). Shunting the venous drainage from the transplant site to the liver completely reversed the diabetic state, probably due to the increased extraction of insulin passing into the liver, indicating the importance of the hepatic portal circulation in islet transplantation (599).

Reece-Smith et al (600) reported that isolated adult syngeneic islets reversed experimental STZ-diabetes in the Lewis rat when implanted under the kidney capsule. However, renal subcapsular allografts were rejected with a mean survival time of

8.4 days. This was comparable to the mean survival time observed for foetal allografts implanted at this site. Interestingly, survival of intraportal adult islet allografts was shorter than for islets implanted under the renal capsule, suggesting that the renal capsule may act as an immunoprivileged transplantation site. This may be explained with respect to the larger number of fixed elements of the reticuloendothelial system in the liver, e.g. macrophages, which may play a dominant role in the rejection of transplanted islets (601). Similarly, isolated adult allografts implanted into the peritoneal cavity, a macrophage-rich site, are also rejected rapidly (588). Hiller et al (602) confirmed these findings and reported that isolated islets transplanted into the portal vein of STZ-diabetic rats showed a progressive deterioration of function with time. In contrast, islets placed under the kidney capsule sustained a long-term function, and controlled all clinical signs of diabetes. Recipients displayed normal growth rate, peripheral serum glucose and insulin levels. However, their functional reserve was markedly reduced as revealed by diminished glucose tolerance and reduced insulin-secreting capacity after an IVGTT.

Late graft failure after intraportal islet transplantation in the rodent model is not a rare occurrence and appears to be strongly related to the site of implantation, as this effect was not observed in kidney subcapsular islet transplantation. Several reasons have been suggested to explain this phenomenon. Histological evidence suggests that the development of fibrosis in the periportal fields of the liver containing the transplanted islets may impair their blood supply, resulting in progressive loss of islet function. Islets have to transmigrate intact vessel walls from a branch of the portal vein into the portal field which may lead to disintegration or destruction. Finally, the portal blood which directly perfuses the intraportally transplanted islets contains higher concentrations of glucose than arterial blood perfusing the kidney and therefore islets transplanted beneath the renal capsule. This may lead to continual stress of the

intraportal islets and their eventual functional exhaustion. Furthermore, two weeks after transplantation, the replication of islet cells with a portal venous drainage was shown to be impaired compared with renal grafts with systemic venous drainage.

Another proposed immunoprivileged site for the transplantation of islets is the thymus. This organ has been reported to be excluded from routine T lymphocyte immunological surveillance, and recirculation of mature T lymphocytes back through the thymic parenchyma is rare. This implicates the existence of a blood-thymus barrier (603). Freshly isolated allogeneic rat islets implanted in the thymic lobes of STZ-diabetic rats survived longer compared with the liver or renal subcapsule as the site of implantation. Upon treatment of the recipients with a single dose of ALS the intrathymic grafts survived permanently (604). Furthermore, a state of donor-specific unresponsiveness was achieved in recipients carrying long-term intrathymic islet grafts, as demonstrated by allowing the survival of a second donor-strain islet allograft transplanted under the contralateral renal capsule. No additional immunosuppression was required and neither grafts were rejected. In contrast, third party islets transplanted under the renal capsule were not accepted. Three weeks after the transplantation, thymectomy did not cause hyperglycaemia. Under histological examination, islets were abundant, non-infiltrated and contained well granulated β-cells. A single dose of ALS transiently depleted the peripheral T cell population to 10% of levels observed in normal rats. Subsequent reconstitution of the peripheral T cell pool in these animals might require the migration of prothymocytes to the thymus where maturation in a microenvironment harbouring foreign alloantigens may induce the selective unresponsiveness observed.

1.6.1.1. PREVENTION OF ALLOGRAFT REJECTION

Although endocrine tissue is generally less immunogenic than other tissues, islet allografts are rapidly rejected in rodents, even across minimal histocompatibility barriers. Three general approaches have been studied to induce islet allograft tolerance : generalised immunosuppression, immunoalteration of the graft prior to transplantation and induction of specific immune tolerance.

1.6.1.1.1. Generalised immunosuppression

A variety of immunosuppressive regimens effective in preventing rejection of vascularized organ allografts in the rodent have been shown to be relatively ineffective in prolonging allograft survival (605). Particularly disappointing results were obtained using the potent immunosuppressive agent CsA. However, Dibelius et al (606) reported that in a one donor-one recipient model, the mean graft survival of intraportally transplanted allogeneic islets could be prolonged from 5 to 90 days using a short term three dose treatment of parenteral CsA. Administration of CsA in this manner resulted in very high concentrations in the blood. Histologic examination at 150 days post-transplantation revealed intact islets that stained positive for insulin. A 3 day peri-operative course of CsA alone did not prolong islet transplantation across MHC barriers (607). However, when combined with a single dose of donor antigen given the day before transplantation, survival time was prolonged slightly, but significantly. Three cycles of 3 day CsA courses at intervals of 7 days (9 doses in total) were found to be effective in delaying rejection of islet allografts. If combined with donor antigen therapy, the survival of islet allografts was further prolonged, and 50% of recipients were still normoglycaemic 60 days after transplantation. The combination of donor antigen with a relatively short course of CsA therefore has a

powerful effect on the prevention of islet allograft rejection. A state of indefinite donor-specific unresponsiveness was not established using this treatment as shown by the rejection of donor skin grafts, and a subsequent return to hyperglycaemia.

Toxic effects of CsA on islets have been reported. Daily administration of CsA (15 mg/kg body weight) to rats caused impaired glucose tolerance, decreased (50%) pancreatic insulin content, reduced (30%) islet insulin content, and decreased (28%) pancreatic β-cell volume compared with vehicle-treated control rats (608). Helmehen et al (609) reported similar observations in the rat after a treatment period of only one week. The pancreatic β-cells are therefore very sensitive to CsA *in vivo*, and the toxic effects appear to be β-cell specific, as the glucagon content of the pancreas was not reduced. In an extensive study, Pipeleers-Marichal et al (610) examined the effect of CsA on the survival of rat islet β-cell allografts in STZ-diabetic rats. A daily oral dose of 5 mg/kg prevented the rejection of freshly isolated islets relatively uncontaminated with exocrine tissue. However, upon discontinuation of CsA islets were rapidly rejected unless cultured prior to transplantation, in order to reduce their immunogenicity. Under these conditions islets maintained a normalised state for >15 weeks beyond the 5 week drug course, although this was not the case for shorter periods of treatment. Pure islet β-cell grafts were found to reverse IDDM in 50% of recipients without treatment of CsA, although mononuclear cell infiltration was markedly reduced after CsA administration. In addition, aggregates of mixed endocrine islet cells, reported by this group to maintain a larger insulin reserve and tighter control of basal glucose levels than pure β-cells (610), were kept virtually infiltration free. Conditions with minimal initial infiltration were associated with long-term graft survival without the need for continuous immunosuppression. It was hypothesised that CsA suppressed the mild T lymphocyte alloreactivity toward the cultured or pure donor islets, thus allowing the generation of

antigen specific suppressor T lymphocytes. Once developed, these suppressor T lymphocytes may have induced a state of tolerance toward the graft that persisted even after cessation of the treatment.

Recently FK506, a neutral macrolide antibiotic extracted from the fungus Streptomyces *tsukubaenis*, has been shown to prolong islet allograft survival in the STZ-diabetic rat (611). Relatively pure islets were transplanted either beneath the renal capsule or into the liver via the portal vein of diabetic rats. Recipients were then treated for 7 days with subcutaneous injections of 0.1-1.0 mg/kg doses of FK506. The optimal dose of FK506 for the prolongation of rat islet allograft (mean survival time of >45.3 days) was 0.32 mg/kg. Success was only achieved when islets were transplanted into the liver via the portal vein, and not under the renal capsule. Histologically, islets from the livers of long-term normoglycaemic animals were intact and contained well granulated β-cells. Foci of mononuclear cells were only occasionally observed adjacent to the islet grafts. FK506 has an entirely different molecular structure to CsA but has the same mode of action, and weight for weight is ten times more potent than CsA, and less cytotoxic. However, FK506 is also toxic to islets when used at high doses, but not at the low dose (0.32 mg/kg) used in this study to prolong islet allograft .

1.6.1.1.2. Immunoalteration

In 1957, Snell (612) suggested that passenger leukocytes within transplanted organs may be responsible for the initiation of immune rejection, because of their mobility. Such cells could carry graft antigen along lymphatics to the regional lymph nodes where the response to foreign antigen is initiated. However, antigen on the surface of dead cells is relatively weak immunogenically (613) leading to the notion that

alloantigen is only highly immunogenic when presented on the surface of active APC. If treatment of the graft prior to transplantation removes active APC, immunogenicity will be reduced. Snell's hypothesis has been further extended : class II MHC antigen expressed on certain mesothelial cells within islets may induce rejection, whereas the endocrine epithelial cells lacking class II antigen are not immunogenic. Dendritic cells within the interstitium, monocytes, and to some extent endothelial cells are class II positive, and these cells are assumed to be the specialised leucocyte population primarily responsible for the induction of the immune response against an allograft. A number of ways of pretreating islets with the aim of reducing their immunogenicity have been demonstrated in rodent models, which generally involve destruction or alteration of passenger lymphoid cells without affecting the islet endocrine cells.

Lafferty et al (614) prevented rejection of thyroid allografts in mice by culturing the donor thyroid in almost pure oxygen atmosphere for 3 weeks pretransplantation. The high oxygen tension destroys passenger lymphoid cells in the donor thyroid, thus hampering antigen presentation and preventing rejection. However, individual rat islets cultured under these conditions only survived 3-4 days, unless clusters or aggregates of approximately 50-150 islets were formed. These islet aggregates, after culture for 7-10 days in 95% oxygen, successfully reversed the STZ-diabetes when transplanted across a MHC barrier as defined by normoglycaemia and aglycosuria (615).

It has been reported that human lymphocytes maintained at 22^{o}C survive and consequently lose their ability to stimulate an MLR *in vitro* (616), suggesting that low temperature culture altered or depleted passenger lymphoid cells. Lacy et al (617) found that *in vitro* culture of isolated rat islets for 7 days at 24^{o}C prior to transplantation into STZ-diabetic rats immunosuppressed with a single injection of

ALS resulted in islet allograft survival of 100 days, even when islets were transplanted across a MHC barrier. Both the period of low temperature culture and the temporary immunosuppression of the recipient were necessary for graft survival, as neither alone prevented rejection. Subsequently, Tze and Tai (618) reported that low temperature culture alone (26^{o}C) for 7 days significantly prolonged the functioning of rat islet allografts when transplanted into non-immunosuppressed STZ-diabetic animals. Islets cultured at higher temperatures (32^{o}C or 37^{o}C) failed to prolong islet survival. Both groups reported that successfully transplanted allografts maintained their antigenicity, i.e. islets were still capable of being recognised as foreign, as an i.v. injection of donor strain peritoneal exudate cells or splenocytes (both a rich source of class II positive cells) induced the rejection of the established allograft.

Another successful approach used in the alteration or depletion of APC is irradiation of donor islets with ultraviolet (UV) light. Lewis rat islets subjected to a dose of 900 J/m^{2} did not exhibit any alteration in their endocrine function, and when transplanted into non-immunosuppressed syngeneic rats, 73% of recipients showed a marked prolongation of survival (>80 days) (619). Lau et al (620) also observed that irradiated rat islets remained hormonally functional and had prolonged allograft survival when transplanted across a relatively weak histocompatibility barrier into a non-immunosuppressed recipient. The islets were reported to contain numerous, although apparently non-functional, intra-islet class II positive passenger leucocytes. This treatment did not prevent rejection when irradiated WF rat islets were transplanted into Lewis recipients, i.e. across a stronger histocompatibility barrier. For strong allogeneic combinations, UV-irradiation in conjunction with a 3 day course of CsA (15 mg/kg) was necessary to markedly prolong survival (18 days) of transplanted allogeneic islets. If the CsA concentration was increased to 30 mg/kg, the combined treatment allowed 100% islet allograft survival for >120 days

demonstrating the effectiveness and synergism between pre-transplant UV-irradiation of the islet allograft and pretransplant immunosuppression of the recipient with CsA in inducing prolonged islet allograft survival in high responder recipients. Singular use of either modality was shown to be ineffective (605).

As mentioned earlier, dendritic cells are thought to be one of the specialised passenger leucocyte populations and elimination of these cells using a specific dendritic cell moAb and complement before transplantation has been achieved with isolated mouse islets (621). However, other laboratories have reported no success using this technique (622,623) raising the possibility that treatment does not always suceed in eliminating all MHC class II positive cells and/or that MHC class II negative cells may also contribute to the immunogenicity of the graft. Unfortunately, a specific dendritic cell moAb for rat is not available.

Dendritic cells express class II MHC antigens and using the same principle, anti-class II antibodies with complement have been used in the pre-treatment of rat islets with a varying degree of success. Reece-Smith et al (622) pre-treated isolated adult rat islets with moAb against class II antigens and complement in conjunction with ALS before their implantation beneath the renal capsule of allogeneic recipients. Treatment only resulted in a slight increase in survival of the islet allografts when compared with controls. This lack of success may be attributed to using antibodies that did not fix complement. Flesch et al (624) used a secondary antibody in conjunction with anti-class II antigen moAb and complement to circumvent this problem. Despite an evident reduction in class II antigen bearing cells within the treated islets, prolongation of graft acceptance after islet allotransplantation across an MHC barrier into STZ-diabetic recipients was not observed. Complete class II antigen depletion within pancreatic islets has also been unsuccessful in prolonging allograft acceptance

(623), suggesting other factors may also be involved in allograft rejection. Terasaka et al (625) found that pre-treatment of donor islets with specific antibodies to class II rat antigens did not prevent their rejection when transplanted into STZ-diabetic Lewis recipients unless used in conjunction with a 3 day course of CsA.

1.6.1.1.3. Induction of tolerance

Evidence from several groups suggest that a state of specific immune tolerance can be induced in recipient rodents bearing an established islet allograft. Transplants of untreated islets from a third party donor into tolerant rats were rapidly rejected, whereas transplants of fresh islets from the original donor strain were retained. The preculture of islets and the immunosuppression of recipients was not necessary, although this treatment was essential for the acceptance of the first pancreatic islet transplant (626). UV-irradiated donor spleen cells (615) or blood lymphocytes (627) could also induce a state of specific immunological tolerance. This pre-immunisation of recipients with class II positive cells allowed the prolonged acceptance and function of untreated islet allografts.

Allogeneic islets were also permanently accepted when transplanted under the renal capsule of adult rats bearing long-term allogeneic kidneys of the same donor strain as the islets, without any further immunosuppression (628). Acceptance of the kidney allograft was induced by a 14 day course of CsA, and 100 days later the recipients were made diabetic with a single dose of STZ. Third party islets transplanted under the renal capsule were rapidly rejected. Similar results were obtained by Gray et al (629) who implanted islets either beneath the kidney capsule or into the portal vein of tolerant STZ-diabetic rats. In both cases, 80% of the recipients showed prolonged

graft survival (>100 days). Thus, once a recipient has accepted a renal allograft under the influence of CsA treatment it will permanently accept an islet allograft of the same donor strain as the kidney, and this effect is not influenced by the site of islet implantation.

Another approach to the specific induction of tolerance was investigated by Liebel et al (630). This group inoculated STZ-diabetic WF rats with minced portions of Lewis rat pancreas initially at a concentration of a millionth of the estimated weight of the whole gland and finishing using a thousandth of the weight. This treatment was given for one year and had no effect on the diabetic state of the recipients as judged by their continued insulin requirement. Transplantation of 500-800 Lewis islets by injection into the portal vein resulted in the reversal of diabetes in 70% of the rats, and these rats remained normoglycaemic until they were killed after 6 months. Histological studies showed the presence of many well granulated islets within the lumen of portal vessels. No regeneration of β-cells in the native pancreas was observed, proving normoglycaemia was due to the implanted islets. Controls were not inoculated with minced pancreas and rejected the allogeneic islets rapidly.

In summary, syngeneic islets transplanted into the STZ-diabetic rat allows the prolonged, if not permanent, survival of the graft at several implantation sites. However, the consistent rejection of islet allografts transplanted into antigenically different diabetic hosts, even across minor histocompatibility barriers, is well established. Decreasing the immunogenicity of the islets by deletion of intra-islet class II MHC expressing passenger leukocytes, i.e. dendritic cells and macrophages, has prevented the rejection of allogeneic tissue and achieved permanent survival of islets in allogeneic hosts. Although chemically-induced diabetic hosts are useful models for the study of immunogenetic and metabolic aspects of islet transplantation, the fact

that IDDM is a disease with an autoimmune pathogenesis necessitates the evaluation of islet transplantation in an animal model with spontaneously occurring diabetes, which has an aetiology more comparable to human diabetes.

1.6.2. **THE BB RAT**

Early transplantation studies showed that MHC-compatible WF islet allografts transplanted intraportally to the spontaneously diabetic BB rat were rapidly destroyed unless the recipients received continuous immunosuppression with ALS (631). Survival was indefinite and histologically the majority of intrahepatic islets contained viable β-cells. Animals normoglycaemic for 6 months after islet transplantation returned to a hyperglycaemic state soon after ALS therapy ceased. Histological evidence of islet rejection was observed, indicating that immunosuppression was necessary to prevent islet allograft destruction. Furthermore, in several long-term islet allograft recipients, both the intrahepatic and native pancreatic islets contained viable β-cells suggesting that ALS therapy not only prevented the rejection of transplanted islets but could also alter the autoimmune response directed against the native pancreas. Like et al (632) found that the injection of rabbit antiserum to rat lymphocytes reversed hyperglycaemia in 36% of BB rats and prevented diabetes onset in prediabetic DP-BB rats. Timely immunosuppression could therefore prevent diabetes in BB rats with sufficient remaining β-cells. However, abnormal glucose tolerance and focal islet lesions indicate that ALS does not completely protect these animals against the cell mediated autoimmune process.

Subsequent experiments investigated the survival time of WF and DR-BB rat islet allografts transplanted intraportally into spontaneously diabetic BB rats or into long-term normoglycaemic DP-BB littermates rendered chemically diabetic by

injection of STZ (633). The immune destruction of islet allografts was rapid in spontaneously diabetic recipients, whereas in 60% of their non-autoimmune STZ-diabetic littermates, grafts survived for up to 300 days before rejection was observed. This data suggests that the autoimmune process responsible for the destruction of native pancreatic islets in the BB rat could also participate in the destruction of transplanted islets. In order to distinguish between autoimmune recurrence of diabetes and rejection in the above studies, rejection had to be precluded. As syngeneic islet transplantation is not possible in the BB rat, DP-BB rats were rendered tolerant to WF histocompatible antigens by inoculation of bone marrow cells at birth, as evidenced by the permanent acceptance of WF skin allografts, and monitored for the development of diabetes. Littermates that did not spontaneously develop diabetes were rendered diabetic by the injection of STZ. WF islet allografts transplanted into tolerant chemically-induced diabetic rats restored permanent normoglycaemia. In contrast, there was a uniform recurrence of hyperglycaemia in the tolerant spontaneously diabetic BB recipients after transplantation of WF islets, which could not result from rejection. This data strongly indicates that recurrent autoimmunity leads to the infiltration of transplanted pancreatic islets by mononuclear cells, resulting in the eventual destruction of β-cells. An analogous response was later observed in human IDDM where recurrent insulitis selectively destroyed islet β-cells following a segmental pancreatic isograft exchanged between identical twins discordant for diabetes (6).

As the autoimmune response in BB rats is thought to be both initiated by and directed towards the native pancreatic β-cells, DP-BB rats tolerant to WF antigens were used to determine if autoimmunity resulted from an intrinsic islet defect or a defect in the immunoregulatory system of BB rats (634). The development of diabetes in DP-BB rats was found to correlate highly with the degree of lymphopenia (635), and

tolerant BB rats predicted to develop diabetes were treated with STZ during the prediabetic period in order to destroy their native β-cells. Transplantation of a WF islet allograft did not prevent onset of disease despite the replacement of native β-cells with non-diabetes prone islets. Furthermore, diabetes onset occurred at approximately the same time as that expected of unmanipulated DP-BB rats. This data indicates that anti-β-cell autoimmunity is not the result of an intrinsic islet defect but to a defect elsewhere, such as the immune system of the BB rat.

Evidence points to a T lymphocyte mediated destruction of pancreatic β-cells in the BB rat and antigen recognition by T lymphocytes has been demonstrated to be MHC-restricted, requiring antigen to be associated with self MHC molecules *in vitro* (636). Indeed, early experiments by Naji et al (637,638) found that MHC-incompatible islet allografts transplanted into diabetic BB rats made tolerant to donor antigens at birth were permanently accepted, providing evidence that this hypothesis may indeed be the case. It was therefore suggested that the autoimmune process destroying native islet β-cells might not be able to destroy islets of a different MHC haplotype.

Woehrle et al (639) confirmed that freshly isolated islets transplanted into the liver via the portal vein of the spontaneously diabetic BB rat were destroyed whether the islets were MHC compatible or not. Preculture of islets for 14 days at 24°C in conjunction with a single dose of ALS at the time of transplantation was necessary to allow permanent survival of MHC-incompatible but not MHC-compatible islet grafts. This demonstrated that the autoimmune rejection of transplanted islets could be avoided by tissue culture if the recipient and donor differed at the MHC locus. In the case of MHC-compatible islet allografts, factors other than recurrent autoimmunity may contribute to islet destruction since *in vitro* cultured MHC-compatible islets were

also destroyed when transplanted into chemically-induced (non-autoimmune) diabetic BB recipients. APC depletion was only successful in preventing allograft rejection if donor recipients were disparate at the MHC locus. This has been verified by transplanting other endocrine tissues such as thyroid and parathyroid tissue (640,641). It has been proposed that when MHC-compatible APC-depleted grafts were transplanted to hosts, the recipients APC population was able to effectively present graft antigens to appropriately restricted T lymphocytes, thereby initiating a rejection response (642). Antigen(s) from an APC-depleted MHC-incompatible graft when processed by host APC have the capacity to stimulate T lymphocytes specific for graft antigen(s) only in the context of self MHC. However, the same antigenic determinants present on the islet allografts are expressed in the context of the donor foreign MHC and hence are not recognised or destroyed by effector cells. It should be noted that after the development of diabetes in the BB rat such hosts have already generated and possess primed T lymphocyte effector populations that developed during the destruction of their native β cells. Logically the restriction in such hosts is more likely to be at the level of the effector cell-target cell interaction than at the induction phase of the immunity. Thus, the survival of APC-depleted MHC-incompatible islets but not MHC-compatible islets in diabetic BB hosts could be explained by the fact that effector T lymphocytes generated against native islets are specific for islet antigen(s) in the context of $RT1^u$ and hence are not capable of interacting with and/or damaging non-$RT1^u$ expressing islet grafts.

Furthermore, Markmann et al (643) examined relative vulnerability of MHC-compatible and -incompatible islet allografts to autoimmune damage after transplantation to the liver via the portal vein of the diabetic DP-BB rat. Rejection in these animals was circumvented by either induction of islet donor-specific immunological tolerance or by pretransplant *in vitro* culture of islets to deplete

intra-islet APC. In the first situation, MHC-compatible and -incompatible islet grafts were equally susceptible to autoimmune damage, reportedly due to increased peripheral blood NK cell activity. NK cells are cytotoxic to islets *in vitro* and may play a role in the non-MHC-restricted diabetogenic response *in vivo*, therefore both MHC-restricted and non-MHC-restricted mechanisms are capable of contributing to anti-β cell autoimmunity and destruction in BB rats. In the latter case MHC-incompatible grafts were significantly less vulnerable to autoimmunity than MHC-compatible grafts, suggesting some degree of protection. This protection was not absolute and varied depending on the donor strain used, pointing to an MHC discriminating autoimmune process. This conclusion was further supported by the recent work of Hegre et al (644,645) who isolated neonatal rat islets using a non-enzymatic technique. These islets are known to exhibit reduced immunogenicity when transplanted to allogeneic diabetic BB rats because of an absence of MHC class II positive cells. MHC-matched islet grafts were still susceptible to disease recurrence in contrast to MHC-mismatched grafts, despite the destruction of the β-cell population of the endogenous pancreas. All MHC-mismatched grafted animals showed ameliorated conditions and 75% of recipients were restored to a normoglycaemic state, i.e. recurrence of diabetes was not observed. Possibly the culture process that rendered the islets allotransplantable in this study also altered the expression of the islet-specific antigen on the β-cell surface responsible for the initiation of the autoimmune process. This target antigen may have been present in animals in a high enough concentration to allow autoimmune damage or destruction of MHC-matched WF grafts but not in the remaining animals. Conversely, grafted MHC-mismatched islets may not bear the diabetic antigen in high enough concentrations to precipitate autoimmune destruction.

Furthermore, the endocrine components of neonatal islets increase in mass after transplantation, and if sufficient tissue is initially transplanted, diabetes may eventually be reversed. Neonatal islet grafts from diabetic BB rats were also found to be larger than grafts from non-diabetic controls and the increase in mass was attributable primarily to proliferation rather than hypertrophy. High glucose levels in the diabetic BB recipient are known to stimulate β-cell replication in foetal, neonatal and adult islets (646-648) and may be the stimulus for enhanced graft growth in this model. A small immunomodulated neonatal graft may therefore increase 10-fold in mass when placed in the diabetic environment of the BB rat, thus leading to the amelioration of the symptoms of diabetes.

Chabot et al (649) demonstrated that combination of UVB donor islet treatment and pretransplant host CsA immunosuppression led to the indefinite survival of MHC-incompatible islet allografts, thus avoiding the reinitiation of the original autoimmune process. Woehrle et al (639) reported that MHC-incompatible islets precultured for 14 days at 24^{o}C and transplanted intraportally into diabetic BB rats treated with a single dose of ALS at the time of transplantation survived long-term. In contrast, survival of treated MHC-compatible islets was similar to that of untreated MHC-compatible islets, suggesting the autoimmune destruction of transplanted islets can be avoided by tissue culture alone if the recipient is briefly immunosuppressed and differs from the donor at the MHC locus.

Two reports contrast the previous studies concluding that destruction of transplanted islets in the diabetic BB rat is MHC-restricted. Prowse et al (650) reported that allotransplantation of cultured MHC-incompatible islet and pituitary tissue under the renal capsule of diabetic DP-BB rats resulted in tissue specific destruction of grafted islets but not of pituitary tissue, despite a pretransplant culture period to reduce

immunogenicity of the islets. Weringer and Like (651) confirmed that immune insulitis in the DP-BB rat was not MHC-restricted in a similar study. Both MHC-compatible and -incompatible islet grafts were destroyed by the disease process responsible for damage to the animals native islets, as evidenced by recurrent lymphocytic insulitis in the transplanted grafts. This process was tissue-specific since islet α- and δ- cells were relatively undamaged. These studies suggest that cultured islets retain the antigen specificity necessary for autoimmune attack, and effector cells responsible for induction of insulitis do not have to share the MHC of their target cells. In addition, β-cells do not need to be antigenically abnormal as a prerequisite for autoimmune attack and, as class II antigen positive cells were presumably destroyed on donor islets by tissue culture, donor class II positive cells appear unnecessary to initiate insulitis in grafted islets of the DP-BB rat.

Reece-Smith et al (600) demonstrated that the renal capsule is an immunoprivileged site compared to the liver. Woehrle et al (652) compared the kidney capsule as a site for islet transplantation with the liver, via the portal vein, in spontaneously or STZ-diabetic BB rats. Both sites are highly vascularized and have been shown to allow good metabolic efficiency of transplanted islets in the STZ-diabetic rat. MHC-compatible and -incompatible islet allografts were found to survive significantly longer when the renal capsule, but not the liver via the portal vein, was used as the site of transplantation without host immunosuppression or islet pretreatment.

Freshly isolated islets transplanted intraportally or under the renal capsule were rapidly rejected. Pretransplant culture for 12-14 days at 37^{o}C induced a slight prolongation of allograft survival at these sites. However, culture at 22^{o}C for the same period significantly delayed and even prevented rejection, but only when islets were placed under the kidney capsule. Upon nephrectomy of the islet bearing kidney,

an abundance of well preserved β-cell clusters were observed histologically. In contrast, rejected islets showed an almost complete destruction of grafted tissue and demonstrated many mononuclear cells infiltrating and replacing the transplanted islet tissue. In a more recent study, Woehrle et al (653) confirmed their previous findings using non-diabetic DP-BB donor islets transplanted under the renal capsule or intraportally into spontaneously or STZ-diabetic BB rats, without immunosuppression or immunomodulation of the donor islets. Earlier studies showed that intracolony BB rat skin grafts were permanently accepted. Islets transplanted into STZ-diabetic DP-BB animals survived indefinitely (>200 days) irrespective of the transplant site. Histologically, islets were found to be intact and stained positive for insulin. Islets also survived long-term when transplanted beneath the renal capsule of spontaneously diabetic BB rats. In contrast, islets transplanted into the liver, via the portal vein, of DP-BB rats only maintained normoglycaemia for 6-14 days before returning to a state of hyperglycaemia. Body weights of animals decreased, and total destruction of islets was observed due to the recurrence of autoimmune insulitis. Although in agreement with earlier studies, these results were in direct contrast with the findings of Prowse et al (650) and Weringer and Like (651), who observed recurrent autoimmune insulitis after transplantation of both MHC-compatible and -incompatible islets under the renal capsule of the DP-BB rat. Woehrle et al suggested that several factors might explain these differences, including incomplete prevention of islet allograft rejection and transplantation of a number of islets insufficient to maintain proper metabolic control.

Posselt et al (654) also looked at the renal capsule and the liver as sites for the transplantation of islet grafts in the newly diabetic BB rat, and compared the results with those obtained using the intrathymic site. The survival of the MHC-compatible and -incompatible islets transplanted under the renal capsule of the DP-BB rat was

significantly prolonged (65 days), compared with islets transplanted intraportally which failed after a mean of 9 days. However, both MHC-compatible and -incompatible islets were permanently accepted when transplanted to the thymus, without the need for chronic immunosuppression. In addition, recipients bearing established Lewis MHC-incompatible intrathymic grafts for >120 days were given a second Lewis islet transplant intraportally. These recipients remained normoglycaemic even after the removal of the primary intrathymic graft and the thymus showed well granulated, non-infiltrated islets. A return to hyperglycaemia was only observed following the removal of the liver 60-65 days after injection of the second graft. The results from these studies show that the intrathymic site not only prevents rejection of transplanted MHC-compatible and -incompatible islets in the DP-BB rat but also protects the islets from destruction by recurrent anti-β-cell autoimmune disease. Furthermore, recipients with established intrathymic grafts failed to destroy subsequent donor strain islets transplanted to extrathymic sites, suggesting that mechanisms which modify systemic allogeneic and autoimmune responses are involved. The deletion or functional inactivation of alloreactive clones followed by the extensive repopulation of the T cell repertoire by newly matured tolerant T lymphocytes has been proposed.

In a related study, Posselt et al (655) investigated the effect of intrathymic inoculation of a small number of MHC-compatible allogeneic islets (not enough to reverse adult diabetes) at birth in the DP-BB rat. Both autoimmune disease and pancreatic insulitis were prevented. This phenomenon was islet-specific, as evidenced by a similar incidence of lymphocytic thyroiditis in both islet-treated and control animals, and was not due to a global impairment in T cell development, as intrathymic and saline controls had comparable lymphocyte numbers and phenotypic profiles in peripheral lymph nodes. The pancreas was found to be healthy and contained viable

islets with no mononuclear cell infiltration. However, thyroid biopsies taken at thymectomy showed severe lymphocytic infiltration in 50% of recipients. In contrast, islets grafted under the renal capsule failed to significantly decrease the incidence of diabetes, despite the persistence of the transplanted islets, demonstrating that islets have to be inoculated directly into the thymus in order to influence anti-β-cell autoimmunity. As the thymus plays an important role in T cell self tolerance induction (656), intrathymic inoculation of islets in the neonatal period exposes T cell precursors to β-cell specific autoantigen(s) resulting in the selective deletion or inactivation of autoreactive clones from the nascent T lymphocyte repertoire. This effect could be mediated by interactions of thymocytes with implanted islets expressing the β-cell autoantigen-MHC complex, or with host-derived thymic APC bearing processed β-cell antigens shed from the islet graft. Alternatively, long-term residence of islets in the thymus could stimulate the selection of specific regulatory cells capable of suppressing the anti-islet autoimmune response.

Brayman et al (657) obtained similar results when uncultured MHC-incompatible islet allografts were transplanted intrathymically to 4-6 week old (i.e. prediabetic) DP-BB rats. No immunosuppression was administered and none of the DP-BB rats became hyperglycaemic, compared with 100% incidence of diabetes in controls. The normoglycaemia persisted for >8 months and all rats had a normal IVGTT. Thymectomy after 241 days did not precipitate hyperglycaemia, confirming that intrathymic islet allografts preserved native β-cell function at an early age, i.e. before puberty. Furthermore, thymuses containing an intrathymic islet allograft were twice the normal size of thymuses of untreated DP-BB rats suggesting an alteration in the natural progression of thymic atrophy, a known consequence of insulin deficiency. Both insulin and insulin growth factor I have been reported to restore the weight of the thymus in diabetic rats towards normal (658).

1.6.3. INDUCTION OF TOLERANCE USING MONOCLONAL ANTIBODIES

The essential function of the immune system is to distinguish self from non-self, and this discrimination is primarily a function of T lymphocytes. Activation of T cells by self antigens is under stringent control involving both thymic (659) and peripheral mechanisms (660). Failure of these mechanisms to control tolerance to self antigens may lead to autoimmunity.

Ideally, therapeutic immunosuppression administered over a short-term period would achieve long-term unresponsiveness to a desired antigen without impairing the host's response to infectious agents. As discussed earlier, currently available immunosuppressive drugs are inadequate since they are relatively non-antigen specific, exhibit limited efficacy, require long-term administration and incur a sustained risk of infection and undesirable side effects. If it were possible to re-establish tolerance in an autoimmune disease or to guarantee tolerance to a transplanted allograft, then it might be possible to dispense with conventional long-term drug immunosuppression. However, achieving tolerance induction as a therapeutic goal in autoimmunity or in transplantation requires an understanding of the development of self-tolerance.

1.6.3.1. T CELL RECOGNITION OF NON-SELF

T cells recognise foreign or non-self antigen as peptide fragments displayed to them in the clefts of MHC class I and II molecules which are expressed on the surfaces of certain cells of the body. In addition, T cells that possess unique clonally distributed receptors respond to these antigens displayed on APC in lymphoid tissue, proliferate

and differentiate to effector mode. Dendritic cells have the greatest ability to present antigen and activate T cells as they possess a particular array of cell surface ligands complementary to an array of adhesion molecules on T cells, and also express MHC class II in abundance (661). A further requirement for T cell activation is one of collaboration or help from other T cells responsive to the same antigens (662).

T cells, once activated, develop into cells whose differentiated function can be that of cytokine release, thereby influencing the functions of other cells. B lymphocytes and pre-cytotoxic T lymphocytes are stimulated to become antibody-producing plasma cells and cytotoxic T cells respectively, and NK and inflammatory cells are activated. The microenvironment in which T cells engage their antigen can determine the type of cytokines secreted, e.g. IL-2 and IFN-γ play a prominent role in IDDM.

1.6.3.2. T CELL RECOGNITION OF SELF

The bulk of self tolerance induction occurs within the thymus where immature T cells with anti-self receptors engage self antigens and undergo clonal deletion as an obligate process. The ancillary mechanisms that induce tolerance in T cells to peripheral antigens are less well defined and probably arise through clonal deletion and anergy, i.e. inactivation but survival of T cells (663). There are currently four possible explanations for tolerance induction following recognition of self antigen which are not mutually exclusive:

a. T cells may still be exquisitely susceptible to tolerance soon after leaving the thymus.

b. The first T cell to engage peripheral self will do so without any collaboration from other T cells therefore the encounter cannot be registered. Consequently,

antigen-reactive T cells could never accumulate in sufficient numbers to mount a response.

c. Much antigen presentation would occur on non-professional APC that lack critical ligands present on dendritic cells and macrophages. Antigen seen in this context might also be tolerance permissive.

d. The context of antigen presentation may also guide the T cell response to be regulatory, protective or suppressive by directing it towards a commitment to produce particular cytokines.

1.6.3.3. MONOCLONAL ANTIBODIES TO ACHIEVE THERAPEUTIC TOLERANCE

MoAb have been investigated as potential immunosuppressants due to the large number of surface molecules involved in T cell interactions with APC and T cells. They can be used as agents to kill defined cell types (depleting) or to interfere with the function of a particular receptor (non-depleting). As they must be administered systemically and are potentially immunogenic, moAb are generally administered over a short-term period only.

There are two strategic approaches based on self-tolerance : the first involves use of moAb to bring about central, i.e. thymic, tolerance, which requires a two-stage attack. Initially, peripheral T cells must be inactivated prior to the introduction of a permanent source of antigen, which can access the thymus and thereby induce tolerance in subsequently developing T cells. The second strategy involves the establishment of a form of peripheral tolerance that would be long-lasting, even after moAb therapy is stopped. Maintenance of tolerance would depend upon antigen continuing to engage the immune system.

Perhaps the most potent moAb investigated are those directed against $CD4^+$ and $CD8^+$ T lymphocytes, targeted because of the central role these T cell subsets play in the induction of immune responses. CD4 antigen correlates to a subset of mature helper/inducer T lymphocytes that recognise antigen on APC in the context of class II gene products. CD4 is also present on the surface of most developing rat thymocytes (664) and on macrophages (665). The functional role of CD4 appears to be in enhancing T cell activation by serving as an accessory molecule for the antigen-specific T cell receptor (TCR) (666). However, it is not yet clear whether CD4 functions simply as an accessory molecule, which stabilises the interaction between the TCR and its specific antigen/MHC class II target, or whether CD4 itself has an active role in signal transduction. CD8 antigen correlates to the cytotoxic/suppressor T lymphocyte subset that recognise antigen on APC in the context of class I MHC gene products and is additionally found on rat thymocytes and NK cells.

Qin et al (667) reported that peripheral (post-thymic) T cell tolerance was achieved in mice injected with the foreign protein antigen human γ-globulin under cover of a short course of non-depleting anti-CD4 moAb. Tolerance was lost in the absence of further exposure to antigen as new T cells were exported from the thymus which had had no contact with the antigen. Tolerance to skin and bone marrow grafts differing at multiple minor transplantation antigens additionally required anti-CD8 moAb treatment. Under these circumstances tolerance was permanent, presumably because the established grafts acted as a continuous source of antigen to reinforce the tolerant state. In both cases, tolerance was not broken by infusion of unprimed spleen cells. More recently, conditioning with anti-CD4 and anti-CD8 moAb allowed the successful transplantation of allogeneic bone marrow in the immunocompetent adult mouse when donor and recipient were mismatched at multiple minor

histocompatibility loci or at MHC class I plus minor loci, but not where the mismatch involved the entire MHC (668).

Gutstein et al (669) found that treatment of mice with moAb to L3T4 (a cell surface glycoprotein expressed on mouse T helper/inducer lymphocytes) blocked both primary and secondary immune responses, delayed allograft rejection and retarded autoimmunity. Tolerance was also induced when anti-L3T4 moAb and anti-chicken ovalbumin moAb, but not anti-T200 (an antigen expressed on all mononuclear blood cells) moAb, were given concurrently, indicating that treatment with moAb to L3T4 may induce tolerance to some but not all antigens. Koike et al (670) used moAb to show that the surface phenotypes of >90% of infiltrated cells in pancreases of NOD mice were $Thy1^+$ and $Lyt1^+$ T lymphocytes, including $L3T4^+$ T lymphocyte subsets. Administration of the L3T4-specific rat anti-mouse IgG2b moAb, GK1.5, to 2-week-old NOD mice twice weekly for 12 weeks prevented the development of insulitis and diabetes, suggesting an essential role for $L3T4^+$ T lymphocytes in the pathogenesis of IDDM.

Short courses of anti-CD4 and anti-CD8 moAb were also shown to induce tolerance to allogeneic skin grafts (a demanding test of any tolerance protocol) in mice (671). Tolerance could be obtained without T cell depletion across multiple minor antigen mismatches both in naive and primed animals demonstrating that peripheral T cells could be tolerised even if they had been activated previously. When donor and recipient were incompatible across the whole MHC, a combination of depleting followed by non-depleting antibodies was necessary to achieve tolerance, as neither treatment alone was successful. This group further demonstrated that mice given a single short course of anti-CD4 and anti-CD8 moAb became tolerant to

MHC-incompatible vascularised heart allografts in a donor and organ-specific manner (672).

Blocking (non-depleting) moAb were equally effective demonstrating that T cell depletion was not obligatory for tolerance induction. Either therapy alone was sufficient to establish tolerance although anti-CD8 moAb therapy was associated with poor recipient survival. A second donor-type heart allografted to the neck of recipients carrying long-term heart allografts (>120 days) was also accepted. However, donor type skin allografted at 100 days was sometimes chronically rejected and third-party skin grafts mismatched at the MHC were always rejected indicating that tolerance induction was donor and tissue specific. Madsen et al (673) also used subset-specific moAb directed against the L3T4 (CD4) and Lyt-2 (CD8) differentiation antigens in order to evaluate their effects on murine cardiac allograft rejection in both naive and primed recipients. Treatment with an anti-L3T4 moAb effectively prolonged the survival of H-2 and non-H-2 mismatched cardiac grafts in naive but not skin graft-primed recipients. This group had previously shown that GK1.5 also significantly prolonged cardiac allograft survival in similar murine strain combinations (674). In contrast, anti-Lyt-2 treatment had no effect on allograft survival in naive recipients bearing a cardiac allograft but did delay graft rejection in recipients primed for donor alloantigens. These data suggest that the $L3T4^+$ ($CD4^+$) T cell subset primarily mediates the first-set rejection response to whole organ allografts, whereas the $Lyt\text{-}2^+$ ($CD8^+$) subpopulation plays an important role in the second-set allograft rejection.

Shizuru et al (675) investigated the conditions under which allogeneic pancreatic islets survived when transplanted intraportally into MHC-disparate STZ-diabetic mice. Islets survived indefinitely in recipients receiving a single i.p. course of GK1.5 moAb

at the time of islet allograftment. Treatment selectively depleted >90% of L3T4$^+$ T cells long-term, thereby allowing the survival of islets without additional immunosuppression. However, tolerance was broken and long-term surviving islet grafts rejected upon i.p. administration of donor-specific spleen cells. These findings suggest that the T helper/inducer lymphocyte subset, as defined by the expression of L3T4 molecules, is central to the induction of allograft rejection.

The rat has also been used to investigate induction of tolerance to cardiac grafts using moAb therapy (676). Treatment of normal rats with anti-CD4 moAb starting on the day of transplant prevented heart graft rejection across a full MHC haplotype mismatch. Adequate doses of OX35 moAb (a very potent anti-CD4 moAb *in vivo*) completely eliminated all peripheral blood CD4$^+$ T cells and induced tissue-specific tolerance to the transplants, as second (fresh) donor heart grafts were retained indefinitely whilst third party grafts were promptly rejected. However, OX38 (an anti-CD4 moAb which binds to a different epitope on the CD4 molecule than OX35) was ineffective in inducing unresponsiveness to neonatal cardiac tissue transplanted into MHC-mismatched congenic rat strains, despite eliminating all peripheral CD4$^+$ T cells. Herbert and Roser also report that an intact thymus is necessary for the development of a population of suppressor T cells in anti-CD4 moAb-treated animals which are responsible for the induction of tolerance. However, skin grafting to high-responder recipients required CsA treatment in addition to administration of anti-CD4 antibodies for tolerance induction. Previously, these authors used a quantitative assay for heart graft rejection to show that the first set rejection of a full MHC haplotype-mismatched graft depends exclusively on CD4$^+$ T cells in the rat. In contrast, the accelerated rejection of second-set grafts in specifically immune recipients depends exclusively on CD8$^+$ T cells (677).

Shizuru et al (678) also used OX38 moAb to prolong the survival of heterotopic vascularised rat heart allografts transplanted across MHC barriers in the rat. Using fluorescence-activated cell sorter (FACS) analysis, administration of a maximal depleting level of OX38 moAb was shown to selectively deplete 80-95% of $CD4^+$ T cells from peripheral blood of treated rats, whereas Herbert and Roser (676,677) reported complete depletion of $CD4^+$ T cells using this moAb. Additionally, animals given moAb doses corresponding to maximal depletion did not make a humoral response against the administered immunoglobulin, whereas those treated with less than maximally depleting amounts made a dose-dependent humoral immune response against the mouse anti-rat moAb. Another potentially undesirable consequence of anti-CD4 therapy might be elimination of immunologic memory, thus rendering a treated recipient vulnerable to a wide range of previously encountered common immunogens. However, rats allowed to reject a primary heart allograft still rejected a secondary heart graft transplanted under the cover of OX38 treatment, indicating that memory responses to alloantigen persisted in rats receiving anti-CD4 therapy. More interestingly, and again in contrast to the findings of Herbert and Roser (676,677), OX38 moAb, when given prior to transplantation of heterotopic abdominal heart allografts, promoted long-term survival of the grafts. Furthermore, these rats accepted second donor-strain hearts, but not third party heart grafts, transplanted into the femoral space without further treatment. This anti-CD4-induced unresponsiveness persisted for at least 90 days following surgical removal of the heart allografts and retransplantation of a second donor-matched heart. Untreated rats rejected their heart allografts within 14 days following transplantation. These results show that transient, pretransplant OX38 moAb therapy induced specific, long-lasting unresponsiveness to fully MHC-mismatched cardiac allografts in rats without additional immunosuppression. Preliminary data from this laboratory showed similar results

using the OX35 moAb in an identical pretransplant treatment regimen as that described for OX38.

Seydel et al (679) also studied the effects of anti-CD4 moAb treatment on the survival of allogeneic islet allografts transplanted to STZ-diabetic rats. OX38 when given as a 4-day treatment regimen caused depletion of >80% of $CD4^+$ T cells from peripheral blood. Three days after initiation of OX38 immunotherapy, rats received an allogeneic pancreatic islets graft via the portal vein. The transplanted islets returned the OX38-treated recipients to a normoglycaemic state which was maintained indefinitely in the absence of further immunosuppression. Elimination of $CD4^+$ T cells at the time of transplant was followed by repopulation of $CD4^+$ T cells from thymic precursors that were incapable of inducing allospecific responses against the transplanted tissue. In contrast, treatment of recipient rats with OX8 moAb, which recognises the cytotoxic/suppressor T lymphocyte ($CD8^+$) subset, induced only a slight prolongation of graft survival (approximately 30 days) despite efficient depletion (>80%) of peripheral blood $CD8^+$ T lymphocytes. Furthermore, long-term islet survival following pretransplant OX38 therapy could be abrogated by coincident treatment of recipient rats with depleting levels of OX8 moAb, indicating the necessity for a regulatory $CD8^+$ T cell to induce and/or maintain anti-CD4 mediated islet survival in the STZ-diabetic rat.

Roza et al (680) looked at the effect of several different moAb specific for rat T cell subsets on heterotopic cardiac transplantation in the rat across combined MHC and non-MHC (minor) differences. Administration of OX19 moAb, which recognises thymocytes and all T cells ($CD5^+$), at the time of transplantation and on alternate days thereafter until rejection, significantly prolonged allograft survival when compared with allografts transplanted into unmodified recipients. Administration of

W3/25 moAb prolonged allograft survival only slightly. W3/25 moAb recognises most rat thymocytes, macrophages and the helper/inducer T lymphocyte ($CD4^+$) subset, and competes with OX38 (665) for binding to rat CD4, suggesting that these two antibodies recognise the same epitope, or determinants in the same region of the CD4 molecule. Administration of OX8 moAb had no effect on allograft survival. In contrast, administration of OX8 markedly prolonged allograft survival across non-MHC (minor) differences, whereas W3/25 moAb administration had no effect. The relationship between graft survival and depletion of targeted T cell subsets, as assessed by FACS analysis, was variable. OX8 moAb administration resulted in a marked reduction of $OX8^+$ cells, but no prolongation of graft survival across combined MHC and non-MHC differences was observed. In contrast, indefinite graft survival was achieved across isolated non-MHC differences. OX19 moAb administration prolonged graft survival, and significant numbers of $OX19^+$ cells were present at rejection. Administration of W3/25 moAb prolonged allograft survival across combined MHC and non-MHC differences despite failing to significantly reduce the $W3/25^+$ cell population, which suggests that W3/25 moAb binds to its target cell without subsequent clearing, i.e. W3/25 is a non-depleting moAb.

In contrast, Claesson et al (681) failed to prolong the survival of anastomotic rat heart grafts in a semi-allogeneic system using W3/25 moAb treatment. This discrepancy may be due to differences in the strain of animals used, the degree of antigenic disparity, or variation in the dose and potency of W3/25 moAb used. Furthermore, treatment with the anti-CD8 moAb, OX8, immediately after transplantation of an allografted heart significantly prolonged the mean graft survival. When given prior to transplantation, graft survival was further prolonged, demonstrating that a selective impairment of the cytotoxic/suppressor T cell population *in vivo* could prolong organ allograft survival.

W3/25 and OX8 moAb were also used to prevent autoimmune recurrence in islets allotransplanted intraportally into spontaneously diabetic BB rats made tolerant to donor-specific islets (682). MoAb treatment was administered pretransplant and continued until recurrence of hyperglycaemia was observed or to the end of study at 100 days. None of the OX8-treated islet graft recipients exhibited recurrent diabetes, which strongly implicates the involvement of OX8-bearing cells in the diabetogenic immune response. However, as both NK cells and cytotoxic/suppressor T cells express CD8 it is unclear which specific cell type participates in islet destruction, and contribution of each of these cell types to the autoimmune response requires further study. In contrast, all diabetic BB rats treated with W3/25 moAb exhibited recurrent hyperglycaemia within 47 days of transplantation. This group reported partial effectiveness of W3/25 in eliminating helper/inducer T lymphocytes from the peripheral blood and from lymphoid organs, and did not exclude the possibility of helper/inducer T lymphocyte participation in anti-β-cell autoimmunity.

It should be noted that W3/25 was effective in reversing the disease course of the autoimmune diseases experimental allergic encephalomyelitis (EAE) (683,684) and neuritis (EAN) (685). EAE is induced in rat by injection of purified myelin basic protein. W3/25 has previously been shown to inhibit MLR *in vitro* by acting on responder cells without killing them (686), and prevent *in vitro* activation of cells that mediate the transfer of EAE from sensitised donors to naive recipients (687). In order to determine if W3/25 moAb could inhibit the *in vivo* immune response to EAE, a single i.p. or i.v. dose (1-2mg) of W3/25 was injected into animals 12-13 days after disease induction. Treated animals recovered from EAE within 2 days, whereas control animals exhibited signs of EAE for a minimum of 4 days. W3/25 had no perceptible effect on the number of $W3/25^{+}$ T cells in the peripheral blood indicating that its inhibitory effect is not mediated by simple clearing or coating of responding

cells with immunoglobulin since OX19 moAb (which reacts with all T cells including W3/25$^+$ T cells) treatment given under the same regimen failed to affect the time course of EAE. It was noted that W3/25 moAb took 24 hours to terminate EAE and may therefore have interfered with T cell activation at an early stage. In support of this hypothesis, injection of W3/25 moAb did not prevent EAE development in rats given *in vitro* stimulated sensitised lymphocytes. In addition, Waldor et al (684) determined the importance of immunoglobulin isotype in successfully treating EAE by using IgG1, IgG2a or IgG2b W3/25 moAb. Although all isotypes had identical binding capacities for rat CD4$^+$ T cells, IgG1 and IgG2a W3/25 moAb were superior to the IgG2b isotype in the treatment of EAE, demonstrating that immunoglobulin isotype does play a role in the treatment of this autoimmune disease. In agreement with previous findings, none of the W3/25 isotypes substantially depleted CD4$^+$ target cells *in vivo.*

For the induction of EAN, rats were injected with peripheral nerve myelin and Freunds adjuvant. Subsequent OX8 moAb treatment completely eliminated CD8$^+$ T cells from peripheral blood and lymphoid organs and exaggerated the disease symptoms, suggesting that cytotoxic T lymphocytes only play a minor role as effector cells in EAN and have a suppressive function. However, additional mechanisms that downregulate neuritogenic immune reactions must exist in this model since, even in the maximally OX8-treated group, the clinical symptoms of EAN disappeared spontaneously a few days after onset. In contrast W3/13 (pan T cell reactive) moAb completely prevented the onset of disease, even though treatment only caused a partial elimination of its target cells. In addition, injection of W3/13, but not OX8, moAb led to a diminished responsiveness of spleen cells from treated rats to allogeneic stimulation *in vitro*, whereas OX8 injection caused a complete elimination

of the *in vitro* cytotoxic response to allogeneic cells in the MLR-activated spleen cell population.

Finally, Like et al (688) reported that treatment of DP-BB rats with OX8 or OX19 moAb achieved stable reductions of splenic and peripheral blood NK and T helper/inducer cells respectively, and in addition protected against diabetes. In contrast, none of the anti-CD4 moAb (OX35, OX38 and W3/25) tested depleted the $CD4^+$ T cell population in the BB rat or had a significant effect on the course of the disease. This group therefore concluded that it is the subsets of rat lymphocytes that express CD8 and CD5 surface antigens which participate in DP-BB pancreatic β-cell autoimmune destruction.

The conflicting results reported by different groups with respect to success of tolerance induction have been attributed to several factors, including differences in rat strain combinations used, animal housing conditions, intrinsic differences in moAb structure, and differences in antibody preparation, dose and treatment regimens.

1.6.4. EFFECT OF ISLET TRANSPLANTATION ON DIABETIC COMPLICATIONS IN ANIMAL MODELS OF IDDM

Chemically-induced STZ-diabetes in the rat induces secondary complications of kidney, eye and nervous system similar to the changes observed in the human. Transplantation of a sufficient number of isolated islets from isogenic donors inhibited or reversed these lesions. After 2-3 months, STZ-diabetic rats begin to develop renal lesions with severe morphological changes, including enlargement of the mesangial space, thickening of the capillary wall, vacuolisation of the tubular epithelial cells, protein casts, and deposition of immunoglobulins in the glomeruli. Following the i.p.

transplantation of pancreatic homogenates containing a high number of islets to the STZ-diabetic rat, Mauer et al (689) observed the inhibition and reversal of early lesions within the kidney glomeruli. In subsequent studies, islet transplants were found to decrease mesangial thickening in diabetic rats, but no effect on basement membrane thickening in the glomeruli of these animals was observed (690). Federlin and Bretzel (691) reported similar morphological changes after the intraportal transplantation of 600 pure islets. A decrease in circulating basement membrane antigens and the normalisation of the increased activity concentrations of glucosyltransferase (an enzyme involved in basement membrane synthesis) were also observed in the kidney. However, the degree of reversal of diabetic lesions after islet transplantation was lower in animals with a longer duration of diabetes, suggesting that an early period of reversibility exists which should not be exceeded.

The retinal capillaries of the eye are leaky in STZ-diabetic rats. Early diabetic vessel changes consist of an altered fluorescein-blood barrier and increased accumulation of i.v.-administered dye into the vitreous and the anterior chamber of the eye 10 days after induction of diabetes. Krupin et al (692) used ocular fluorophotometry to demonstrate that leakage returned to normal baseline values when insulin levels were normalised 13 days after the transplantation of islet isografts. Intraportal or intracerebral pancreatic islet allografts completely prevented the characteristic basement membrane thickening in retinal capillaries of the STZ-diabetic rat during a 400-day post-transplantational study (693). In another long-term study, alloxan-diabetic rats transplanted with a whole pancreas were killed at intervals of 1-2 years and kidney and eye pathology compared with age-matched diabetic control rats. Lesions were fewer and less severe in pancreas-transplanted rats than in diabetic control rats, and differences between the lesions in the two groups were highly significant at 20-24 months (694).

Studies of the articular cartilage of diabetic rats revealed a significant increase in the activities of enzymes engaged in the synthesis and degradation of mucopolysaccharides (695). Transplants of islets into the diabetic recipients reversed the increased enzyme activities to normal values.

Abnormalities in the function of the autonomic nervous system may be manifested by cardiovascular, genitourinary, sudomotor and alimentary dysfunction (696). Schmidt et al (697) reported autonomic neuropathy involving the colon wall and the ileal mesenteric nerves in chronic STZ-diabetic rats. Ultrastructural studies of the mesenteric nerves revealed a distinct morphological alteration, apparently reflecting aberrant or frustrated axonal regeneration. Transplantation of pancreatic islets 6 months after induction of diabetes, by which time mesenteric axonopathy was well developed, quickly re-established normoglycaemia. Within 3 months, resolution of the axonal neuropathy was almost complete. Lacy (698) observed the dilatation of the colon (megacolon) after 4-5 months of STZ-diabetes, most probably due to autonomic diabetic neuropathy. Megacolon could be prevented by the transplantation of isogenic islets one month after induction of disease.

Due to the nephrotoxic effects of STZ used to induce diabetes, the effect of early islet transplantation on the prevention of nephropathy is better investigated in the spontaneously diabetic BB rat (207). 1000-1200 non-diabetic DP-BB rat islets were transplanted under the renal capsule of DP-BB rats 1-3 weeks after diabetes onset. At 9 months of age, the islet-bearing kidney was removed from the diabetic recipient and examined for glomerular mesangial deposits and vacuolization of epithelial cells. The amount of mesangial deposits increased slightly with age in the non-diabetic DP-BB rat and no Armanni cells were visible. In contrast, the mesangial deposits were doubled in the diabetic DP-BB rat receiving no islet graft, and >60% expressed

Armanni cells to a high degree. After islet transplantation these parameters decreased to levels observed in the non-diabetic rat. In addition, body weight, blood glucose and HbA_1 values were also similar to non-diabetic control animals.

Islet cell transplantation in the abdominal testis of the BB/Wor male rat normalised fasting glucose and HbA_1 in 56% of rats for a period of at least 6 months. Improved metabolic control was associated with normal total urinary protein, sural nerve morphometry, and sexual function (699).

1.6.5. **HUMAN ISLET TRANSPLANTATION**

1.6.5.1. HUMAN ISLET ISOLATION

Gray et al (700) first described the successful isolation of islets from the human pancreas in 1984. Prewarmed collagenase was injected via the pancreatic duct to distend and dissociate the pancreas into a cell suspension from which islets could then be purified. However, the collagenase digestion step was difficult to control and produced variable results, thus leading to an automated method for human islet isolation as described by Ricordi et al (701). This method involves the distension of the pancreas with collagenase followed by its placement into a digestion chamber through which medium recirculates at 39°C. The chamber is then gently shaken until islets appear, at which point the temperature of the circulating medium is reduced to 15°C and the effluent collected at 4°C. Thus, released islets are immediately removed from the collagenase solution, thereby preventing overdigestion. Islets are then purified by density gradient centrifugation using Ficoll (702) or BSA (703) and can be kept in tissue culture for up to two months. 2000-3000 islets/g of tissue with purities of 60-80% have been reported (701,703,704).

1.6.5.2. TRANSPLANTATION STUDIES

Recurrent insulitis and β-cell destruction have been observed in segmental pancreas grafts transplanted from non-diabetic to diabetic identical twins (14). This recurrent autoimmune destruction and subsequent return of IDDM was prevented by an immunosuppressive protocol similar to that given to patients undergoing pancreas or kidney allografting (705). By 1983, 159 islet tissue allografts had been reported to the International Pancreas Transplant Registry, but none of the patients had achieved insulin independence that could be attributed to the grafts alone (706). No documented transplantation of purified adult human pancreatic islets was given, and graft preparation techniques, immunosuppressive protocols and transplantation sites were highly varied. Potential sites for islet transplantation include the renal subcapsular space, the spleen and intraportal injection into the liver. All of the successful human islet transplants recently reported have used the intraportal site.

The first report describing short-term insulin independence after islet transplantation came from Scharp et al (702). This transplant failed after 20 days probably due to rejection. Since then, Alejandro et al (707) have performed five islet transplantations in four CsA-immunosuppressed ABO-compatible patients with type I IDDM. Islets were isolated by a double collagenase injection technique and purified using volume reduction of the dispersed tissue by centrifuging at slow speeds. Grafts of 5-6 ml, comprising 20-40% islets, were pretreated in four instances with anti-class II moAb and percutaneously transplanted into the portal vein. This report shows that donor islets can be safely engrafted in the liver of patients without the need for general anaesthesia. One patient received a combined kidney/islet transplant from the same donor, while all the other patients receiving an islet transplant had established renal allografts. After islet grafting, three patients demonstrated normal basal plasma

C-peptide levels for several weeks following, but not before, islet transplantation. However, there was only a blunted response to a mixed-meal challenge. One patient, who received an islet transplant on two occasions, showed subnormal C-peptide levels for 18-26 weeks post-transplant. All patients continued to require exogenous insulin treatment after islet transplantation.

Several factors may have contributed to the failure of this series of islet transplants. Islets were isolated from cadaver donor pancreases at least 6 hours after excision, and delay in islet isolation from the time of pancreas removal has recently been shown to be detrimental to islet viability (708,709). In addition, the islet grafts may have been insufficiently pure (20-40%) using the volume reduction technique. Finally, therapeutic levels of CsA were inconsistently achieved.

Since 1990, there have been several encouraging reports of sustained islet graft function after transplantation into diabetic patients (702,710,711). These successful transplants include two diabetic patients that became insulin independent after islet transplantation, one for more than 6 months (711). All reports describe islet isolation from human cadaver pancreases by some form of ductal collagenase injection and Ficoll density gradient purification.

Warnock et al (710) transplanted 260 000 purified allogeneic islets isolated from two pancreases into the portal vein of two IDDM patients synchronously with a kidney from the same donor. Immunosuppression was induced with antilymphoblast globulin, azathioprine and prednisolone; cyclosporin was only introduced 8 days after islet transplantation. Islet cell secretory responses were observed for up to 10 weeks after transplantation, with C-peptide levels rising from negligible levels before transplantation, to half the minimum level that has been found after successful

vascularised pancreas transplantation. Although graft function sufficient to allow withdrawal from insulin was not achieved, this report provides evidence for a sustained β-cell secretory response after combined islet/kidney allotransplantation in an IDDM patient.

Scharp et al (702) reported the first IDDM patient, with an established kidney transplant on basal cyclosporin immunosuppression, who was able to eliminate the requirement for insulin after human islet transplantation. Approximately 800 000 islets were successfully isolated from 1.4 cadaver pancreases that were 95% pure and contained 121 U of insulin. After 7 days of 24^{o}C culture, the islets were transplanted into the portal vein under local anaesthesia, followed by 7 days of antilymphoblast globulin administration and maintenance of the cyclosporin. Blood glucose was kept under strict control via i.v. insulin for 10 days post-transplantation, after which insulin therapy was stopped. The average 24-hour blood glucose level remained <8.3 mmol/l and the C-peptide values, although initially rising slower, exceeded the normal range after an oral glucose load. Twenty-five days after islet transplantation exogenous insulin therapy was reinstated possibly due to a marked stress response (a family death) or a rejection of part of the transplanted islets. Interestingly, some of the islets were cryopreserved for a time prior to transplantation using dimethysulphoxide as cryoprotectant. Tissue recovery after thawing is approximately 80%, and advantages of this storage include detailed matching of tissue, banking and transport of tissue between centres, and pooling of tissue from multiple donors.

Under unusual circumstances, Tzakis et al (711) described nine patients who became diabetic after upper abdominal exenteration and liver transplantation for extensive tumours, and were subsequently given islet grafts obtained from one or two

pancreases. At the time of publication, the longest survivor required neither parenteral alimentation nor insulin, more than 6 months after transplantation.

Two important factors are likely to have contributed to these successes. Firstly, the mass of islet tissue transplanted exceeds that previously reported, and islet grafts were obtained from two pancreases in the majority of cases. Although the longest survivor in the report from Tzakis et al received an islet graft obtained from only one pancreas, the endocrine volume was relatively high (711). Secondly, in the latter two reports, islets were transplanted under conditions that made the rejection of islet tissue less likely. Evidence suggests that an islet allograft may be less immunogenic in the presence of a renal allograft or when combined with a liver allograft from the same donor strain (712). This last circumstance accords with the report from Tzakis et al, in that 8 of 9 patients received at least some islets obtained from the pancreas of the liver donor (711).

Thus, islet transplantation may have the potential of restoring normal or nearly-normal glucose homeostasis in the IDDM patient. In comparison with vascularised pancreas transplantation, this treatment could be applied early in the course of diabetes when diabetic micro and macroangiopathy may still be modified. Steady progress has been made in the techniques of islet isolation, and improvements continue to be made (701,704), bringing the goal of successful clinical islet transplantation closer. However, immunological problems such as rejection and autoimmune destruction of the islet graft necessitate the need for more effective immunomodulation protocols for human islets, such that diabetic patients do not face a lifetime on immunosuppressants. A potential method of circumventing this latter problem involves immunoisolation of the islets in a bioartificial pancreas.

1.7. THE BIOARTIFICIAL PANCREAS

The ultimate goal of islet transplantation is to treat diabetic patients without generalised immunosuppression and early enough in the course of IDDM to prevent or retard the development of complications associated with the disease. The bioartificial pancreas offers a distinct advantage in this respect. The principle of this system involves immunoisolation of non-syngeneic islet tissue, thereby allowing their transplantation into non-immunosuppressed recipients. Immunoisolation systems have been conceived that separate islets from the host by an artificial membrane that is permeable to low molecular weight substances such as glucose, insulin, electrolytes, oxygen and bioactive secretory products, but not to immunoglobulins and other inflammatory cells (713,714). Additionally, these systems offer a solution to the problem of human islet procurement by permitting the use of xenogeneic islets isolated from animal pancreases.

1.7.1. TYPES OF BIOARTIFICIAL PANCREAS

Extravascular systems include islets placed inside diffusion chambers, sealed hollow fibres or microcapsules, all of which may be implanted inside the peritoneal cavity of the recipient. In vascular devices, the islets can be distributed in a closed compartment surrounding a semipermeable membrane, and the device implanted as a shunt in the vascular system. All devices must fulfil certain requirements including the ability of the system to respond to a glucose load by increasing insulin production, thereby correcting diabetes, and the efficiency of the membrane to provide immunoprotection for the islets, so that they survive and remain functional *in vivo*. In addition, when considering human implantation of a bioartificial pancreas, size, safety and biocompatibility are important limiting factors.

1.7.1.1. DIFFUSION CHAMBERS

Diffusion chambers consist of a ring separating two pieces of Millipore filter, forming a chamber in which islets are placed. Early studies by Gates and Lazarus (715) investigated the use of this system, seeded with rabbit neonatal pancreas, to reverse diabetes after implantation into STZ-diabetic rats. Blood glucose, plasma insulin and oral glucose tolerance were all normalised. Upon their removal 6 weeks after implantation, all animals returned to a state of hyperglycaemia, and the neonatal pancreatic tissue from within the recovered chambers was shown to secrete insulin, glucagon and pancreatic polypeptide *in vitro*. More recently, Ohgawara et al (716) reported that allogeneic islets encapsulated within a Millipore chamber, consisting of two nucleopore membranes of pore size 0.6 μm sealed with an O-ring, normalised plasma glucose for up to 3 months after implantation into STZ-diabetic mice.

This approach was reassessed in studies investigating the *in vitro* kinetics of glucose and insulin release from chambers using different membrane materials (717). The diffusion of insulin from chambers of different chemical composition, but similar pore size, differed considerably. In contrast, variation of membrane composition appeared to have little effect on the diffusion of glucose, which was much faster than that of insulin due to the difference in their molecular size. Pore size did not appear to produce significant differences in the rates of insulin or glucose diffusion when membranes of similar chemical composition were used to construct chambers of different porosity. Additionally, it was noted that even with the best membranes, the half-times for transit of glucose into the chambers (14 minutes), and of insulin out (54 minutes) were relatively long, possibly due to the large dead volume inside the chamber. Thus, although islets transplanted inside diffusion chambers may maintain an adequate long-term insulin output, they are unlikely to maintain accurate

minute-by-minute regulation of blood glucose concentration. Indeed, this group (718) further described that 1100-1400 pancreatic islets enclosed in polycarbonate diffusion chambers failed to reverse diabetes after i.p. transplantation into syngeneic STZ-diabetic rats. Upon recovery at the end of the 12 week study, chambers were found to be overgrown with a fibrous capsule comprising of fibroblasts 10-15 cells thick. Furthermore, viable islet tissue was not recovered, although this was not solely due to fibrosis reducing the permeability of the chambers, as viable islets were also not retrieved from freshly implanted devices. However, Theodorou et al (719) demonstrated that diffusion chambers were at least effective in preventing contact of the graft with the inflammatory cells responsible for initiating immune responses, since 1200-1500 allogeneic rat islets encapsulated in polycarbonate membrane in chambers of 0.45 μm pore size and implanted into STZ-diabetic rats produced no detectable anti-islet antibodies in the recipients.

More recently, Lanza et al (720,721) described a series of experiments that use wider-bore tubular membrane diffusion chambers with an internal diameter of 1.7-4.8 mm. Amicon XM-50 membranes used in these studies had a smooth outer skin and a nominal cutoff of 50-80 kD. Porcine, canine and bovine islets placed within these chambers restored normoglycaemia in STZ-diabetic rats for >150 days without immunosuppression (720). Only minimal tissue reactivity was observed, and the external membrane surfaces were generally free of fibrotic overgrowth, exhibiting only occasional host cell adherence. Encapsulated canine xenografts implanted in spontaneously diabetic BB/W rats were equally successful, resulting in fasting normoglycaemia for >1 month in all animals (721). IVGTT *K* values, i.e. the decline in blood glucose levels (%/min), improved 10 days after implantation in BB/W rats and after 1 month in STZ-diabetic rats. Both light and electron microscopy of

long-term functioning grafts revealed well preserved islets, with hormone-producing α-, β- and δ- cells.

In conclusion, wider-bore XM membranes have solved many of the early problems associated with diffusion chambers, including fibrosis, maintaining viability of the islets within the chamber, abscess formation and adhesion of host cells (717,722). Results from preliminary experiments in totally pancreatectomised, severely diabetic dogs, i.e large animal models more closely resembling human diabetes, indicate that canine islet implants can provide long-term correction of hyperglycaemia without the use of immunosuppressive drugs (723). Insulin independence has been achieved for >10 weeks in dogs with preimplantation insulin requirements of >38 U/day. Little or no fibrosis has been observed for periods as long as 30 weeks.

In view of these encouraging results, other unsolved issues that are crucial to the clinical success of diffusion chambers must be addressed, including membrane breakage and further improvements in glycaemic control.

Many studies involving diffusion chambers fail because, under stress, chambers can bend leading to fracture of their membrane walls and subsequent destruction of encapsulated islet tissue. An acute and/or chronic inflammatory response ensues, possibly caused by the broken membranes themselves or by the release of islet tissue from the ruptured chambers. A modest increase in the membrane wall thickness or a decrease in chamber length will produce a stronger chamber and possibly resolve this problem.

Lanza et al (724) observed a 4-fold average increase in insulin secretion over baseline levels upon perifusion of encapsulated canine islets with high-glucose

medium, and the insulin concentration in the perifusate increased after a delay of only 7 minutes. This increase in insulin delivery is comparable to the lag time observed using an artificial pancreas in humans, and will thus avoid overexcursion of postprandial blood glucose concentration (725). More recently, wider-bore chambers were implanted i.p. into pancreatectomised dogs (723) and the responses to i.v. and oral glucose determined. A progressive deterioration of blood glucose control was observed, possibly reflecting membrane chamber breakage or inadequate secretory capacity secondary to a loss of islet cell viability and/or insulin secretory function. In addition, all immunoisolated islets lacking intimate vascular access must be supplied with oxygen and nutrients by diffusion from the nearest blood vessels. In wider-bore membrane chambers, the problem of cell death or dysfunction as a result of oxygen and nutrient supply is likely to be more severe.

1.7.1.2. SEALED HOLLOW FIBRES

In this bioartificial pancreas, islet tissue is placed inside semi-permeable hollow fibres, and the ends sealed prior to implantation. Archer et al (726) initially evaluated the efficacy of implanted hollow fibres (50 kD) containing cultured xenogeneic mouse or inbred isogeneic hamster islets in the reversal of STZ-diabetes in the Chinese hamster. Both islet types returned recipients to a state of normoglycaemia for 46.8 and 52.3 days in xenografted and isografted animals respectively. A second transplant using a larger number of islets after the reappearance of the diabetic state also resulted in long-term normoglycaemia (142 and 230 days respectively), confirming the absence of immune responsiveness to the xenogeneic tissue. However, a fibrous envelope was observed around grafts upon recovery, 3-6 months after implantation. This fibrotic layer is thought to play a critical role in determining the functional capacity of the graft.

Altman et al (727) subsequently reported that permselective tubular Amicon XM-50 hollow fibres (1 mm internal diameter) seeded with human insulinoma fragments normalised non-fasting plasma glucose and insulin levels in 50% of STZ-diabetic rats for up to 1 year, when implanted i.p. These animals showed a comparable weight gain over a 12 month period as control rats. Removal of the hollow fibres led to recurrence of hyperglycaemia, thus demonstrating that the encapsulated insulin-secreting tissue was responsible for the long-term correction of diabetes in these animals. Histological analysis of recovered implants revealed a fibrous tissue layer surrounding the membranes. However, insulinoma tissue retrieved from fibres was shown to be functionally active by electron microscopy studies, and in one case, by perifusion studies after removal of the hollow fibre at 6 weeks. In addition, cataract development and diabetic glomerulopathy were completely prevented in successfully grafted animals (728). This group also reported the positive effect of implanting a hollow fibre seeded with human islets in NOD mice (729). 50% of recipients achieved complete and long-term (>3 months) correction of hyperglycaemia. Moreover, splenocytes isolated from normoglycaemic transplanted NOD mice were able to transfer diabetes upon injection into irradiated non-diabetic NOD males. This finding suggests that the immune process is still active in these mice, thus reinforcing the fact that hollow fibres can protect the graft not only against immune rejection, but also against the diabetogenic autoimmune mechanisms.

Icard et al (730) extended these studies to diabetic and non-diabetic pigs. The same type of fibre was observed to elicit an inflammatory pericapsular response when implanted i.p. or in various other sites, including the area surrounding the splenic vein, the epiploic chambers in the omentum, the external wall of the stomach and the abdominal muscles. Implantation sites possessing a portal drainage showed a milder fibrotic reaction than the other sites studied. The tissue reaction was generally more

intense, though qualitatively similar to that seen in the rat, except for lymphoid clusters with giant and pseudoepithelioid cells that were observed only in pigs. The reaction consisted of several layers (<50 μm in the peritoneum to >100 μm in the abdominal muscles) of fibroblasts and collagen with polymorphonuclear leukocytes, macrophages, histiocytes and small lymphocytes. The fenestrated outer wall of the tubular membrane was always infiltrated by collagen, fibroblasts and macrophages. Despite this intense fibrosis observed in the pig, metabolic success of immunoprotected grafts was obtained, stressing the importance of the quality of the grafted tissue.

Zekorn et al (731) demonstrated the importance of the chemical nature and molecular cut-off of the hollow fibre membrane with respect to the insulin diffusion rate. Furthermore, this group (732,733) failed to correct diabetes in STZ-diabetic mice and rats on a long-term basis using several different types of hollow fibre for the macroencapsulation of islets, including Amicon XM-50 which had successfully been used by Altman et al (727) to completely reverse the STZ-diabetic state in rats. In the former studies, a correction of diabetes lasting only 3-4 days was observed. These different results may have been due to the use of different insulin-producing tissues seeded in the hollow fibre. Zekorn et al (732) also described the influence of the hollow fibre membrane surface on the tissue reaction of the host : membranes with a cavernous surface induced the formation of a thin fibrotic layer and allowed an inward spreading of capillaries and tissue into the interspaces of the wall structure, which might have a beneficial effect on islet survival and function. In contrast, a thicker fibrotic layer was observed around fibres with a smooth surface (734). Again, the protective effect provided by the membrane against the cytotoxic effect on islets of IL-1β was demonstrated (735).

1.7.1.3. MICROENCAPSULATED ISLETS

Another possible approach to avoid rejection in islet transplantation studies is the microencapsulation of isolated pancreatic islets in a biocompatible and semipermeable membrane.

Evidence that the membrane provides efficient immunoprotection for the encapsulated islet tissue was demonstrated by Darquy and Reach (736) who assessed the immunoisolation of rat insulinoma RINm5F cells by microencapsulation using a ^{51}chromium cytotoxicity test. Significant ^{51}chromium release was observed when free (non-encapsulated) islets were incubated with complement and either the serum of a rabbit immunised with RINm5F cells or the sera of two recently diagnosed IDDM patients. This effect was not observed when islets were encapsulated demonstrating successful immunoisolation from anti-islet complement-fixing cytotoxic antibodies by microencapsulation. Soon-Shiong et al (737) further demonstrated the efficacy of the microcapsule in protecting target cells against specific cell-mediated destruction involving cytotoxic T lymphocytes, and non-specific killing by NK cells and NK cytotoxic factor. Again using the ^{51}chromium-release assay, significant cell lysis was observed when pancreatic and tumour cell targets were incubated with free effector cells, or with NK effector cells co-encapsulated with NK-sensitive target cells via the release of NK cytotoxic factors. This effect was not observed with encapsulated effector cells alone.

Lim and Sun (738) first reported the transplantation of microencapsulated islets into the peritoneal cavity of chemically-induced diabetic animals to correct the diabetic state. Basically, isolated rat pancreatic islets were suspended in a sodium alginate solution and extruded through a needle in contact with an air flow, thus generating

microcapsules containing islets. Capsules dropped into a calcium chloride solution causing alginate to gel and form calcium-alginate microbeads. The negatively charged gel capsules were successively coated with the positively charged amino acid poly-L-lysine, followed by an outer layer of polyethyleneimine to mask the bioincompatible poly-L-lysine. Finally, the islets were incubated in sodium citrate buffer and the unbound alginate within the capsule was liquified by removal of the calcium ions.

The viability of the islets after microencapsulation was determined *in vitro* in perifusion experiments. Insulin secretion from microencapsulated islets was comparable to free (unencapsulated) islets, and upon raising the glucose concentration from 2.8 to 16.6 mmol/l there was a biphasic insulin response from both groups of islets. However, a delayed response in insulin secretion was reported in the microencapsulated islets, and this was attributed to the time required for the passage of insulin across the capsular membrane into the medium. The increased secretion of insulin in response to elevated glucose concentrations clearly demonstrated that the viability and function of the islets were retained throughout the process of microencapsulation. Furthermore, microencapsulated islets remained morphologically and functionally intact, and had a normal degree of β-cell granulation when cultured for up to 13 weeks at 37^{o}C. Using immunoperoxidase staining the presence and location of the four pancreatic hormones were also found to be normal (738).

This group also demonstrated that free islet allografts implanted i.p. into STZ-diabetic rats only survived for 6-8 days as expected, whereas recipients of microencapsulated islets were normoglycaemic for almost 3 weeks. The symptoms of diabetes, including polyuria and polydipsia, were markedly reduced during the normoglycaemic period.

The failure of the encapsulated islets was attributed to an inflammatory response induced by the bioincompatibility of polyethyleneimine, and not to allograft rejection.

In two similar studies (739,740), multiple i.p. transplants of approximately 3000 islets encapsulated in alginate-poly-L-lysine-polyethyleneimine capsules were injected into STZ-diabetic rats. Initial transplants lowered blood glucose concentrations to control values, but after 20 days the animals returned to a diabetic state. A second transplant of microencapsulated islets again lowered the blood glucose to within the normal range and this pattern was repeated until the animals were killed 90 days after the initial islet transplants. A control group of STZ-diabetic rats received no transplants or transplants of empty capsules and had blood glucose levels >19.4 mmol/l throughout the study. The body weights of the control STZ-diabetic rats did not change significantly over the period of study and eye cataracts developed within 12 weeks of diabetes onset. In contrast, the two animals receiving multiple islet transplants increased in body weight and remained cataract free. Upon sacrifice, histological studies revealed that capsular membranes were completely surrounded by giant cells and fibrous tissue whether they contained islets or not. No intact microcapsules or viable islets were recovered from the recipients and failure of the microencapsulated islets was again attributed to the poor biocompatibility of the outer polyethyleneimine layer.

Subsequently, Sun et al (739) modified the composition of the microcapsule membrane by replacing the outer polyethyleneimine coating with a more biocompatible alginate layer. The thickness of the poly-L-lysine layer was also increased in order to strengthen the capsule. Untreated STZ-diabetic control rats given single injections of free islets were normoglycaemic for less than 10 days. In contrast, a single transplant of 4500 modified islet-containing capsules restored

normoglycaemia in all recipients within 2 days. This state persisted for up to 10 months, demonstrating the immunoprotective properties of the alginate-poly-L-lysine-alginate (APA) membrane. Good control of diabetes was observed during the normoglycaemic period, and recipients steadily gained weight, excreted normal volumes of urine and showed no development of eye cataracts. A second transplant of 5000 encapsulated islets upon regression to the diabetic state again reversed diabetes, and 80% of recipients remained normoglycaemic for a significantly longer period than was observed after the first transplant, demonstrating that simple allograft rejection was not the cause of graft failure. 50% of free capsules recovered from the abdominal cavity were found to be intact with surfaces free of cell attachment, and contained viable islets which secreted low levels of insulin *in vitro* in response to a glucose challenge. Remaining capsules also appeared intact but had fibroblast-like cells several layers thick on their external surfaces. Electron microscopic studies revealed essentially smooth capsule membranes and the thickness of the wet wall suggested that the capsule wall was a hydrogel containing 90% water. Hydrogels are good biocompatible materials which reduce frictional irritation to surrounding tissues and cause minimal protein interaction with the aqueous biological environment thereby contributing to their biocompatibility.

The variation observed in the life span of the individual transplants is probably due to the variable number of functioning β-cells. If layers of fibroblast-like cells build up on the surface of capsules, the rate of diffusion of nutrients, glucose and insulin across the capsule membrane would be slowed and death of some islets may result. Upon transplant failure, the number of surviving β-cells is insufficient to maintain glucose homeostasis but may prolong the life span of a subsequent transplant by increasing the total number of functional β-cells.

Cole et al (741) looked at the metabolic effects, namely intermediary metabolites, diurnal blood glucose and HbA_1, of allogeneic microencapsulated islets implanted into the peritoneal cavity of STZ-diabetic Wistar rats. Transplantation of approximately 3000 microencapsulated islets normalised blood glucose concentrations for 4 weeks, before a return to hyperglycaemia at 6 weeks. Blood concentrations of the ketone 3-hydroxybutyrate was also corrected during the 4 weeks of normoglycaemia, showing that the grafts were effective in reversing ketosis normally associated with uncontrolled hyperglycaemia in IDDM. Both these metabolic parameters remained abnormally elevated in control STZ-diabetic animals implanted with empty microcapsules. No differences in lactate, alanine or glycerol concentrations were observed between the two groups. HbA_1 as an indirect measure of long-term glycaemic control was lower, but not significantly so, in transplanted animals compared with control diabetic animals, and did not return to normal levels. A gradual increase in HbA_1 values was observed in both groups with increasing duration of poorly controlled diabetes. Transplanted animals also showed a marked variation in blood glucose over a 24 hour period, with lower values during daylight hours and nocturnal peaks during the animals normal feeding times. This observed hyperglycaemia probably reflected poor carbohydrate tolerance as suggested by Fritschy et al (742). This group confirmed that normoglycaemia could be achieved after transplantation of microencapsulated rat islet allografts into STZ-diabetic recipients for up to 4 months, in the absence of immunosuppressive therapy. Body weight and weight gain were normalized as was the volume of urine excreted. However, despite persisting normoglycaemia, the kinetic responses to oral and i.v. glucose challenge were abnormal. The low insulin increments during glucose tolerance testing seemed to suggest that the observed non-fasting normoglycaemia was achieved by a basal release of insulin from the encapsulated islet graft. However, glycaemic stimulation of microencapsulated islets has previously been shown to result

in a rapid and significant insulin response when tested *in vitro* (743). Other factors possibly explaining the lack of insulin response by microencapsulated islets to blood glucose elevation after transplantation include the large size of the microcapsules and the total volume of the encapsulated islet tissue implanted, and a gradual decrease in graft viability due to the occurrence of fibrotic overgrowth. Thus, microencapsulated islets grafts did not achieve normal metabolic homeostasis in the chemically-induced diabetic rat despite an amelioration of the diabetic state.

The effect of the capsule size on insulin release by microencapsulated rat islets in response to glucose *in vitro* was further investigated by Chicheportiche and Reach (744). APA capsules were found to range from 300-800 μm in diameter and two groups (n = 10) were used for static incubation studies according to their size. Large (650 μm) or small (350 μm) microencapsulated islets were cultured in medium containing either 5.5 mmol/l glucose (basal medium) or 16.5 mmol/l glucose and 5.5 mmol/l theophylline (stimulatory medium). The insulin concentration of basal or stimulatory medium bathing small capsules rose significantly from the fifth minute onward, whereas the increase in insulin concentration of basal and stimulatory medium bathing large capsules was not significant. Two hypotheses were suggested to explain the absence of a significant insulin response to stimulatory glucose observed in large capsules. Firstly, possibilities for exchange across the microcapsule membrane decrease as the capsule diameter increases, and secondly, islets contained in small or large capsules are of similar size and will secrete similar amounts of insulin in response to glucose. Thus, intracapsular insulin concentration will be much smaller in large microcapsules, since the secreted insulin will be diluted in a volume approximately 8-fold larger. As the driving force for insulin diffusion is the difference in insulin concentration across the membrane, a slower insulin release from the large microcapsules would be expected. In agreement with this hypothesis is the finding

that the magnitude of insulin secretion by free islets in response to glucose was, under similar conditions, 7-fold greater. The volume of the microcapsule has therefore been shown to be an essential parameter in the kinetics of insulin secretion in response to glucose by microencapsulated islets.

Fritschy et al (743) also observed a very poor secretion of insulin by freshly microencapsulated islets in response to glucose stimulation, and the glucose-induced insulin secretion of free islets was 10-fold greater. This observation could not be explained by inadequate permeability of the capsule because insulin release was also severely reduced when islets were subjected to the encapsulation procedure but without the membrane-forming poly-L-lysine step. Therefore, islets were tested *in vitro* after each step of the encapsulation procedure, as originally described by Lim and Sun (738). Insulin release was severely reduced after prolonged suspension of islets in saline or treatment with citrate. Insulin release improved significantly when the saline and citrate steps were replaced by calcium-free Krebs-Ringer bicarbonate buffer and 1 mmol/l EGTA respectively, both with and without complete encapsulation. Interestingly, the capsule membrane as such was found to have no influence on glucose and insulin diffusion, as suggested by Chicheportiche and Reach (744).

Recently, Zekorn et al (745) cross-linked alginate with barium cations to produce a matrix which reportedly was more stable than calcium-alginate (746). 80% of STZ-diabetic mice xenotransplanted with 800 barium-alginate encapsulated rat islets i.p. achieved post-prandial normoglycaemia for the duration of the experiment (28 days). Upon retrieval, barium-alginate beads were surrounded by a thin layer of fibrotic tissue. Siebers et al (747) employed these novel barium-alginate capsules to assess the amount of free and microencapsulated syngeneic islets required to correct

STZ-diabetes in the rat. 3000 free islets transplanted i.p. failed to normalize non-fasting blood glucose levels unless the recipient received a second transplant of 3000 islets. In contrast, transplantation of only 1200-1500 islets beneath the kidney capsule regularly resulted in long term normoglycaemia within 10 days of transplantation. After graft removal by nephrectomy the diabetic state was re-established. These findings are in agreement with the work of Woehrle et al (748) who described the peritoneal cavity as an inferior transplantation site compared with the kidney capsule. However, in the rat model the peritoneal cavity is the only transplantation site that is capable of bearing the volume of microencapsulated islets, although in terms of nutrition and oxygenation its unsuitability is well recognised (749). Nonetheless, 3000 encapsulated islets were able to restore long term normoglycaemia within 10 days of i.p. transplantation in the rat. Empty microbeads did not alter the diabetic state of recipients. The need for an increased free islet mass in these transplantation studies may be explained when considering oxygen supply and cytokine action. Oxygen concentration in the peritoneal cavity is low and free or microencapsulated islets rely on diffusional transport for their oxygenation which in turn is dependent on the membrane surface of the capsule, the diffusion distance, and the diffusion coefficient of the medium. As the barium-alginate gel contains >98% water, the membrane does not represent a significant barrier for oxygen diffusion although the diffusion distance is enlarged in the case of the microcapsules. In addition, the individual microencapsulated islets act as seperate oxygen consuming units which decreases competition for a deficient substrate and moreover, the contact surface is enlarged approximately 20-fold. Cytokines released during any inflammatory process have been shown to be potent inhibitors of islet function and, depending on the type and concentration, can be cytotoxic to islet cells (349-351). Microencapsulation might protect the islets against such attack.

The transplantation of microencapsulated islets into spontaneously diabetic BB rats has been less successful than transplantation into chemically-induced diabetic rats. Only one group has achieved long-term normoglycaemia following implantation of microencapsulated islets (750). Transplantation of 4000-5000 encapsulated islets i.p. reversed the diabetic state of all recipients within 3 days and normoglycaemia was maintained for 190 days without immunosuppression. Normal body weight and urine volumes were maintained during this period and no cataracts were detected in the transplant recipients. Removal of the capsules in one recipient 67 days after transplantation resulted in recurrent hyperglycaemia within 2 days. Approximately 90% of the implanted capsules were recovered and of these, 70% were free-floating, free of cell overgrowth and contained viable islets. The remaining 30% of the capsules had cell overgrowth on their membranes. A second transplant of microencapsulated islets was required by some animals after the reappearence of hyperglycaemia and again a state of normoglycaemia was achieved. Control rats receiving transplants of free islets were normoglycaemic for less than 2 weeks, demonstrating that microencapsulation of islets not only protected allografted islets from graft rejection, but also from autoimmune destruction.

In contrast to this work, Gotfredsen et al (751) reported that diabetic DP-BB rats receiving 5000 MHC-compatible DR-BB rat islets encapsulated in an APA membrane resulted in state of normoglycaemic with a mean duration of only 54 days. Upon recovery from the peritoneal cavity after graft failure, most capsules were shown to be covered by a layer of histocytes and occasional foreign body giant cells, surrounded by concentric layers of myofibroblasts with an abundance of collagen fibres, suggesting a non-immunological mediated foreign body reaction. Islets within these capsules were degranulated and vacuolized, although there was no evidence of lymphocytic invasion of the capsules or infiltration of the islets. Empty capsules

recovered after 15 and 60 days were also overgrown to a similar extent suggesting that bioincompatibility of capsular materials is responsible for the inflammatory response rather than molecules secreted by the islets through the capsule membrane. As expected, animals receiving an i.p. transplant of 5000 free islets reverted to the diabetic state after a mean of 17 days.

Cole et al (752) confirmed these findings and also observed a marked foreign-body reaction around microcapsules implanted i.p. in both diabetic and non-diabetic DP-BB rats. In contrast, little or no reaction was observed around capsules recovered from the STZ-diabetic WF rat. The finding that biocompatibility of APA microcapsules from the same production run was good in WF rats but poor in BB/E rats suggests that the inflammatory response observed cannot be attributed to variations in preparation methods and/or materials used in different laboratories. Implantation under the kidney capsule, a proposed immunologically privileged site, afforded the microcapsules no additional protection.

Various biomedical polymers have been shown to activate macrophages involved in foreign-body reactions which stimulate IL-1β production, a known β-cell toxin (753). The role of cytokines in the failure of microencapsulated islet grafts in the BB/E rat has therefore been investigated (752). Reduced secretion of insulin and progressive islet cell damage were demonstrated when microencapsulated islets were incubated with IL-1β *in vitro* for 9 days, showing that the APA membrane does not exclude cytokines. It is conceivable that the activation of macrophages as part of the foreign-body response to the capsules leads to the release and subsequent penetration of IL-1β across the membrane resulting in islet tissue damage and graft failure.

Wijsman et al (754) also reported that 5000 allogeneic islets encapsulated in APA membranes and transplanted i.p. into diabetic BB/W rats resulted in graft failure within 2 weeks of transplantation in 68% of recipients. Again, graft failure was associated with a dense pericapsular infiltrate that starved the islets of nutrients and oxygen, resulting in islet necrosis. Approximately 5% of empty capsules recovered 23 days after transplantation showed a capsular infiltrate, and this increased to 60% by day 65, strengthening the view that the APA membrane is not biocompatible. 50% of the capsules recovered were free-floating in the peritoneum, the rest were clumped and adhered to the liver and stomach or surrounded by fibrous sacs associated with omentum or fat within the peritoneum. Again, the pericapsular infiltrate was composed of fibroblasts and granulomas with macrophages immediately adjacent to the membrane, 10-15 cell layers deep. Immunohistologically, islets in capsules free of infiltrate were found to be morphologically normal, viable and contained 62% insulin positive cells. Duration of the graft function was reported to be inversely proportional to the density of the capsular infiltrate. Immunosuppression of diabetic BB recipients receiving 5000 allogeneic microencapsulated islets with CsA or dexamethasone both significantly decreased the percentage of capsules surrounded by infiltrate and the amount of capsular infiltrate (although no effect on the infiltrate cell population was reported). The number and function of viable encapsulated islets was also increased resulting in the prolonged, but not indefinite, function of the graft (754). Indomethacin had no effect on graft survival unless used in conjunction with CsA. Mazaheri et al (755) reported similar findings, and additionally observed capsular overgrowth decreased with an increased microcapsule survival. This is in agreement with the observed increase in pericapsular infiltrate upon implantation of microcapsules into newly diagnosed diabetic animals, as opposed to recipients with established diabetes (>5 months), which show a less vigorous autoimmune response.

The stimulus for the pericapsular infiltration of microencapsulated islets implanted i.p. into the spontaneously diabetic DP-BB rat appears to be the bioincompatibility of the outer alginate layer. Wijsman et al (754) in the above study reported that, more specifically, it was alginate with a high mannuronic acid content that initiated the reaction, and its removal resulted in a decrease in the pericapsular infiltrate. Clayton et al (756) encapsulated rat islets in high mannuronic acid alginate and subsequently coated these capsules with either poly-L-lysine alone, poly-L-lysine high mannuronic acid alginate or poly-L-lysine and an outer layer of high guluronic acid alginate. All capsules responded to an *in vitro* glucose challenge in a similar manner. Results from the implantation of microencapsulated islets under the renal capsule of non-diabetic or diabetic DP-BB rats suggested that infiltration was strain-dependent. The most severe reaction was observed in diabetic BB rats, and this response was relatively unaffected by the microcapsule composition. The infiltration reaction to the capsules was also found to be strain-dependent after i.p. implantation. In addition, the reaction was most severe at this site and was affected by capsule composition. An outer layer of poly-L-lysine alone provoked the strongest immune response, and poly-L-lysine subsequently covered by an outer layer of high mannuronic acid alginate resulted in the weakest response. The finding that high mannuronic acid alginate capsules minimise pericapsular fibrosis is in direct contrast to the findings of Wijsman et al (754). Soon-Shiong et al (757) transplanted empty capsules composed of alginate with either high or low mannuronic acid content into the peritoneal cavity of normal Lewis rats and retrieved them 2-180 days post-transplantation. Low mannuronic acid alginate capsules were largely free of cellular overgrowth, whereas 90% of high mannuronic acid capsules were associated with cellular and fibroblast overgrowth 21 days after implantation. Soluble alginate and alginate gels composed of mannuronic acid blocks have been shown to be potent stimulators of IL-1 and TNF-α production, whereas incubation of monocytes with alginate made from guluronic acid

blocks resulted in only a minimal release of cytokines. In addition, Wolters et al (758) reported that empty capsules prepared from purified alginate of medium guluronic acid content were completely free of overgrowth when recovered 4 weeks after implantation in the omentum. Soon-Shiong therefore proposed that commercial alginate containing high mannuronic acid activates the macrophages involved in inflammatory responses *in vivo*, resulting in cytokine production, fibroblast proliferation and eventual overgrowth of the microcapsule. Subsequently, Soon-Shiong formulated microcapsules with purified alginate containing high guluronic acid that had improved biocompatibility (757), mechanical stability, and adequate porosity. These microcapsules were shown to reverse diabetes after i.p. transplantation in the large animal model (759), with long-term (up to 2 years) islet function after transplantation in spontaneous diabetic dogs even after cessation of all immunosuppression (760), suggesting that biocompatible APA microcapsules can be prepared for use in islet transplantation studies.

Soon-Shiong et al (761) recently obtained regulatory and institutional approval for the first human trials using these improved islet-containing microcapsules. Human islets, isolated by standard collagenase digestion from 8 cadaveric donor pancreases, were encapsulated and cultured for 22 days prior to transplantation into the peritoneal space of the patient. The islets were 85% pure and 9957/kg body weight encapsulated islets were transplanted. The patient had established IDDM (30 years), and suffered from severe long-term complications. End-stage renal failure resulted in a living-related kidney transplantation, thus the patient was on low-dose immunosuppressive maintenance prior to and following transplantation of encapsulated islets. A supplemental dose of 5000 encapsulated islets/kg was given 6 months after the initial dose of approximately 10 000 islets/kg, as part of a dose-escalation study.

At the time of publishing (9 month post-transplant), the patient had no adverse effects and maintained a stable daily mean blood glucose concentration, even after discontinuation of exogenous insulin in the ninth month. Basal C-peptide secretion increased with the drop in exogenous insulin requirements, confirming sustained insulin secretion from the encapsulated islets. Proinsulin levels during insulin independence were high, suggesting that 15 000 islets/kg was sub-clinical, and that islets were being stressed to maintain insulin independence. HbA1 values decreased from 9.3% pre-transplant to 7.6% at 4 months and 7.9% at 9 months. In addition, lower extremity peripheral neuropathy and axonal nerve function improved post-transplantation, and the patient reported increased energy, an ability to walk further, and a general feeling of improved health.

Although immunoprotection of capsules under conditions of low-dose immunosuppression has been demonstrated, further trials on non-immunosuppressed patients are needed to determine the immunoprotection of the capsules without immunosuppression, as well as establish the optimum human i.p. dose of encapsulated islets required to achieve insulin independence.

The apparent success of encapsulated islets in achieving insulin independence in the IDDM patient renews concerns about difficulties in obtaining sufficient supplies of donor human pancreases. In the above study, 8 donor pancreases were required for the treatment of one patient. Alternative sources of pancreas tissue, i.e. from larger animals, might have to be considered.

1.7.1.4. MICROENCAPSULATED ISLET XENOGRAFTS

An extremely attractive feature of immunoisolation of islets by microencapsulation is the possibility of using xenograft tissue without the need for immunosuppression of the recipient. Early studies involved the implantation of microencapsulated islets from a donor into a recipient of a closely related species. O'Shea and Sun (762) reported that 1000 rat islets encapsulated in APA membranes and implanted i.p. into non-immunosuppressed STZ-diabetic mice reversed diabetes within 3 days. Animals remained normoglycaemic for up to 144 days with a mean xenograft survival of 80 days, and >80% of the xenografts functioned for >50 days. A second xenograft of 1000 encapsulated islets given after the failure of the initial transplant also had a prolonged survival rate. Survival of rat islet xenografts after encapsulation in alginate-polyornithine membranes was prolonged in STZ-diabetic mice, with a mean duration of 39 days. This was significantly greater than for xenotransplanted free islets which functioned for <14 days. Upon recovery, the polyornithine capsules were more severely overgrown than the poly-L-lysine capsules, confirming that the former capsules were less biocompatible. Graft failure could be attributed to gradual cell death of the islet cells resulting from lack of oxygen and nutrients as the capsules became overgrown.

Calafiore et al (763) reported that canine islets encapsulated in alginate-poly-L-ornithine biomembranes provided a favourable microenvironment for long-term (120 days) *in vitro* culture, with maintenance of β-cell function and a retention of normal insulin secretory patterns. Microencapsulated human islets were also shown to sustain normal insulin secretory kinetics for at least 28 days in culture. Upon implantation into the peritoneal cavity of STZ-diabetic mice, canine islet xenografts were rapidly destroyed unless microencapsulated

in alginate-poly-L-ornithine biomembranes. These microcapsules initially afforded protection against islet xenograft destruction, and prolonged reversal of hyperglycaemia in all STZ-diabetic and some NOD mice was observed. However, microcapsule properties appeared to be variable both within and between various strains of diabetic mice, and the reversal of diabetes was more frequently observed in STZ-diabetic, rather than spontaneously diabetic NOD mice. This difference may be explained by differences in the quality of islet preparations, immunoreactivity of recipients or severity and reversibility of diabetes. Upon retrieval of microcapsules from long-term (120 days) euglycaemic mice, islets were consistently found to contain no viable β-cells. In contrast, β-cells were shown to be present in the native pancreas, suggesting that the initial euglycaemia induced by the implanted encapsulated islets allowed repair or regeneration of islets in the recipients native pancreas. The loss of islets *in vivo* within microcapsules could be a result of specific immune response to islet xenograft antigens, non-specific reaction to putative mediators of inflammation in the area immediately surrounding the capsules or may reflect the inability of a large mass of islet cells to remain viable in the special microenvironment created by the capsule.

More recently, Soon-Shiong et al (764) showed prolongation of discordant islet xenograft function in STZ-diabetic rats after microencapsulation. Encapsulated canine and human islets were implanted i.p. and free islet grafts acted as controls. Low dose CsA therapy was instituted in both groups, and euglycaemia was maintained for 43-123 and 42-136 days after implantation of encapsulated canine and human islets respectively. In contrast, free islets achieved euglycaemia for <2 days.

Hamster islets encapsulated in an APA membrane were shown to secrete insulin in response to secretagogues in short-term *in vitro* culture and maintain a similar level of

insulin secretion as observed for free islets for 28 days (765). No overgrowth of fibroblastic cells was observed inside the capsule even after 70 days of culture. When xenotransplanted i.p. to STZ-diabetic rats, 3500 encapsulated hamster islets did not achieve prolonged normalization of fasting plasma glucose levels of recipients, although microcapsules recovered from a recipient 27 days after transplantation showed some islet viability. Free islets were replaced by massive connective tissue elements and insulin-positive β-cells were almost undetectable 22 days after transplantation.

Lum et al (766) investigated a novel method of encapsulation using an electrostatic droplet generator to produce APA microcapsules of a significantly reduced size (250-350 μm diameter). The smaller capsules were proposed to increase cell viability, allow faster cell response to glucose fluctuations and greater mobility, and reduce the volume of capsules required for transplantation, which is of paticular importance in microencapsulated islet transplantation experiments using mice as recipients. In an earlier study, this group reported that rat islets microencapsulated in much larger capsules (700 μm diameter) restored normoglycaemia in STZ-diabetic mice for an average of 80 days (761). In a concurrent study, rat islets encapsulated in small capsules and transplanted into STZ-diabetic BALB/c mice survived and secreted insulin in recipients for up to 308 days (767). This study investigated the transplant survival of these smaller capsules in the spontaneously diabetic NOD mouse. *In vitro*, both free and encapsulated islets showed comparable responses to glucose challenge in terms of insulin secretion, although the level of secretion from islets encapsulated in the smaller capsules was approximately 20% lower than that from free islets. Using these new capsules, 16 spontaneously diabetic NOD mice received i.p. transplants of 800 encapsulated rat islets. Non-fasting plasma glucose decreased within 2 days of implantation. At 4 to 5 months post-transplantation only

two animals were normoglycaemic, and removal of their capsules resulted in a return to the hyperglycaemic state. Recovered capsules remained physically intact with enclosed islets clearly visible. Furthermore, perifusion studies showed that recovered microencapsulated islets still secreted insulin in response to a glucose challenge, although the amount secreted was lower than that observed for fresh free islets or freshly encapsulated islets. It is possible that some islet cells, especially in the central portion, became necrotic, possibly due to lack of nutrients, leading to graft failure in the majority of recipients. In addition, there may have been a possible gradual degeneration of some islet cells during long-term function. After a second transplantation of microencapsulated islets, normoglycaemia was again restored within 2 days. However, in approximately 30% of recipients, the xenografts functioned for <3-4 months. Failure was partially attributed to improperly constructed capsules, i.e. capsules produced using impure compounds or of an impaired surface smoothness and shape, which could lead to a more severe capsular overgrowth. In control mice, free rat islets remained functional for <10 days.

It is commonly accepted that the failure of transplanted microencapsulated islets is related to a cellular reaction, particularly intense in the NOD mouse. Weber et al (768) attempted to characterize this reaction by xenografting APA microcapsules containing dog or rat islets i.p. into STZ-diabetic and NOD mice. In addition, the immunologic reaction to " concordant " and " discordant " donor islets could be compared. Both microencapsulated dog and rat islets routinely normalized blood glucose in both types of diabetic mice within 24 hours. However, all grafts were eventually destroyed, more rapidly so in NOD mice than STZ-diabetic mice, and microcapsules eliciting an intense cellular reaction contained no viable islets. In prediabetic NOD mice, implantation of encapsulated dog or rat islets resulted in a moderate cellular reaction. Interestingly, empty microcapsules elicited no cellular

reaction in either diabetic or prediabetic NOD mice, suggesting that the reaction was not directed against the microcapsule itself, but against a secreted product of the xenogeneic islets within. Treatment of the recipient with GK1.5 moAb significantly prolonged the survival of both dog and rat microencapsulated islets xenografted to the NOD mouse. Long-term functioning grafts recovered on days 75-95 demonstrated viable islets in capsules free of infiltrate. Flow cytometry showed undetectable $CD4^+$ T cells in the peripheral blood of all moAb-treated NOD mice with functioning xenografts. However, cessation of antibody treatment after 95 days resulted in eventual graft failure in most recipients receiving microencapsulated rat or dog islets. These results indicate that the reaction to microencapsulated xenogeneic rat or dog islets in diabetic NOD mice is helper T cell dependent.

1.7.1.5. VASCULAR DEVICES

This type of implantable bioartificial endocrine pancreas is advantageous over extravascular devices as continuous blood flow permits quick and direct exchange of nutrients and oxygen between the hosts circulating blood and the transplanted islets suspended in the extracapillary space. In addition, it should be possible to access the cell chamber for the removal and replacement of non-functioning islets once a device is implanted. Finally, the vascularised artificial pancreas is devised so that the artificial capillary is enclosed by an outer casing to prevent fibroblast overgrowth on the extracapillary surface.

Initial investigations by Chick et al (769) showed that β-cells cultured in Amicon XM-50 semipermeable hollow fibres, consisting of a thin retentive skin surrounded by an outer macroporous spongy layer, continued to synthesise, store and release insulin. Furthermore, insulin release was readily modulated by altering the glucose

concentration of the perifusion medium circulated through the lumen of the fibres. However, the use of these small diameter fibres with an internal diameter of <1 mm is limited because of the problems associated with clotting and/or the need for systemic anticoagulation. The application of more non-thrombogenic and biocompatible synthetic materials and larger diameter fibres may enhance the survival of diabetic animals implanted with artificial vascular devices. However, the kinetics of insulin release in response to glucose by such a system may be inappropriately slow if the compartment housing the islets is too large (770). Alternatively, decreasing the resistance to flow may decrease the thrombogenic risk, increase flow through the vascular device and improve kinetics of glucose-stimulated insulin release from islets seeded in the device (771).

Due to a drop in hydrostatic pressure, the pressure at the inlet of the fibre used in the vascular device is greater than that in the islet compartment and an ultrafiltration flux from the blood to the chamber occurs. Similarly, the hydrostatic pressure in the second half of the fibre is lower than that of the islet chamber, and a backfiltration flux from the compartment towards the bloodstream is observed (772). In order to utilise this flux, a U-shaped bioartificial vascular device should be considered to overcome the observed time lag in glucose-stimulated insulin release, since the ultrafiltration-backfiltration flux crosses the islet compartment as a short circuit. The blood channel surrounds the islet chamber, which consists of two flat membranes, and blood circulates successively above the first membrane and then in the reverse direction below the second membrane. Reach et al (773) introduced isolated islets into the chamber and perfused the system with Krebs buffer containing high or low glucose concentrations to determine the insulin release kinetics. For up to 1000 islets, insulin release in response to glucose was linearly correlated to the number of islets seeded in the chamber, indicating that insulin did not significantly inhibit its own

secretion in this system. A significant rise in insulin release was observed after 3 minutes, and a maximal response was observed after 10-20 minutes. When the glucose concentration was reduced to a sub-stimulatory level, insulin secretion declined rapidly. The kinetics of this vascular device were therefore much more satisfactory than those obtained with hollow fibre devices. Finally, during glucose stimulation, the insulin concentration was found to be 4-fold higher than the concentration present at the turning point of the blood channel, suggesting that insulin was transferred into the perfusing medium in part by a countercurrent flux of ultrafiltrate crossing the membranes.

Araki et al (774) demonstrated that vascular bioartificial pancreases could release large quantities of insulin in response to glucose. Capillary devices seeded with approximately 50 000 canine islets released up to 20 units of insulin per day when perfused with culture medium containing 16.6 mmol/l glucose, i.e. approximately one-third of the daily production of insulin by an adult human pancreas. Initially the insulin content of the perifusate during the first 1-2 days of culture was low (possibly as a result of β-cell damage during the isolation procedure), before subsequently rising after 3-4 weeks and reaching a stable plateau after 7 weeks of perifusion.

Tze et al (775) seeded a synthetic capillary unit with approximately 1200 allogeneic rat islets, or 3000 xenogeneic rabbit or human islets and implanted these vascular devices into STZ-diabetic rats. Approximately 77% of the rats receiving allogeneic islets only survived for 12-24 hours and the remaining 23% survived 1-11 days. The diabetic rats receiving implantable devices containing rabbit islets survived for up to 4 days, whereas recipients of human islets seeded in a vascular device survived for 8 days. Immediately following implantation, plasma glucose fell to normoglycaemic levels, and a corresponding increase in circulating insulin levels was observed in all

recipient animals. Furthermore, the bioartificial pancreases produced a near-normal plasma glucose and insulin response to an IVGTT, suggesting the feasibility of achieving amelioration of diabetes with allogeneic or xenogeneic pancreatic islets implanted as a bioartificial endocrine pancreas unit.

However, there was still a time lag in the correction of hyperglycaemia in the diabetic recipients following implantation of the vascular device, probably due to the relatively thick walled large-bore artificial capillary fibre used in this implantable unit. Molecular transport depends greatly on the thickness and surface area of the barrier and volume of the extracapillary space, therefore the delay in achievement of glucose and insulin equilibrium between the extracapillary space and the host circulation would result in reactive hypoglycaemia. Units must therefore be constructed with a larger surface area, reduced extracapillary space and a thinner membrane barrier in order to decrease the observed time lag.

More recently, an artificial pancreas device utilising a single, coiled, tubular membrane with an internal diameter of 5-6 mm and a wall thickness of 120-140 μm, has been investigated (776,777). An XM-50 membrane is incorporated within an acrylic housing, and the islet chamber is created in the space between the membrane and the housing. As the XM-50 membrane cannot be sutured, it is connected to standard PTFE graft material of the same diameter, which extends beyond the housing, and is used for anastomosis to the vascular system. The device was seeded with approximately 260 000 allogeneic canine islets and implanted into pancreatectomised dogs. In 50% of animals requiring <20 U of exogenous insulin per day pre-implant, the daily dose of exogenous insulin was significantly reduced or eliminated. However, even when the implant resulted in fasting normoglycaemia, the response to an oral or i.v. glucose challenge was abnormal. Increasing the number of islets seeded in the

vascular device to approximately 320 000 removed the need for exogenous insulin administration for periods ranging from 1 month to >8 months, demonstrating that these implants can be used to treat severe diabetes. However, the response to a glucose challenge remained impaired. Upon removal, histological evaluation of the vascular devices indicated a substantial loss of islet mass, indicating that islets in the implanted device may need replacing. Furthermore, there was no evidence of infiltration by immune cells, suggesting that the membrane was indeed immunoprotective.

1.7.2. CONCLUSION

As mentioned earlier, the concept of the bioartificial pancreas is extremely attractive and the different systems have been shown to meet the functional requirements, including insulin secretion in response to glucose and other secretagogues, long-term survival in an immunoprotected state, and efficiency of the artificial membrane in protecting the islets against immune factors. However, many problems have yet to be solved, the most important one being the biocompatibility of the foreign material used to form the membrane. An inappropriate immune response of the host can induce a foreign-body reaction for extravascular devices, or thrombosis of the blood channel in the case of a vascular system. Only progress in this field will permit the bioartificial pancreas to be used successfully for the treatment of diabetic patients. Interestingly, the same obstacle limits the development of glucose sensors. Concerning the microcapsules formulated in alginate high in guluronic acid by Soon-Shiong, it remains to be seen whether the same immunoprotectivity of islets by the capsules is observed in non-immunosuppressed patients.

1.8. SOMATIC CELL GENE THERAPY IN IDDM

1.8.1. INTRODUCTION

Gene therapy is a novel approach to overcoming the problems of insulin delivery to the diabetic patient. A major problem of pancreatic and islet transplantation as treatments for IDDM is the immune rejection of transplanted tissue. Even if this problem can be overcome using immunosuppressive drugs, tissues are still destroyed by recurrence of the autoimmune process responsible for the destruction of the native islets. Somatic cell gene therapy could resolve these problems of tissue rejection if cells were used from the diabetic patient.

The principle of this treatment involves the insertion of a functional gene into the genome of a target cell such that expression of the introduced gene is expected to have a therapeutic effect. In the case of IDDM, implanted cells will be genetically modified to produce insulin.

1.8.2. TARGET CELLS

Human tissues used in somatic cell gene therapy at present include bone marrow, skin and lymphocytes, all of which can easily be extracted from the body, grown and manipulated *in vitro*. Upon subcutaneous reimplantion back into the patient, the cellular products will be secreted into the systemic circulation (778). In addition, cells could be encapsulated in a biocompatible membrane so that they could easily be removed if any deleterious effects were observed (779) or if transfected cells die and need replacing. Many studies have used haemopoietic stem cells in particular, as bone marrow transplantation is a well established and successful procedure. The gene of

interest is introduced into totipotent stem cells contained in the bone marrow, and after autologous reinfusion, these cells have the capacity for self-renewal throughout the lifetime of the patient. However, to have a sustained effect, the genetic correction must occur at the level of self-renewing pluripotent stem cells so that there can be continued production of progeny cells containing the introduced gene. These cells are difficult to identify and purify and more research is necessary before bone marrow gene therapy can proceed (780). Alternatively, differentiated cells can be used in gene therapy.

1.8.3. METHODS FOR GENE TRANSFER

DNA can be introduced into target cells as a co-precipitate with calcium phosphate (781) or encapsulated within liposomes (782). Alternatively, target cells can be made permeable by electroporation (783), in which a brief electric pulse allows entry of DNA by way of local areas of reversible membrane breakdown. However, all of these methods suffer limitations, including damage and/or death of target cells, and instability and low frequency of integration of DNA. Direct microinjection of DNA using glass pipettes is more efficient than these aforementioned methods, but is limited by the number of cells that can be microinjected over a given time and the need to purify target cell populations.

Retrovirus vectors are more widely used in genetic engineering as they are non-toxic, have a wide host range and infect cells with high efficiency. The virion binds to cell surface receptors and the RNA genome is transferred into the cell where it is copied into DNA by the virus-encoded reverse transcriptase. This DNA copy then integrates into the host genome.

The use of retroviruses to transfer genes into mammalian cells carries several disadvantages and potential risks, including host cell damage by the insertion of the vector into an essential gene, the activation of a silent proto-oncogene by introduction of viral promoter sequences, or the activation of latent viruses encoded by the genome (784). Improvements in retroviral vector design may eventually solve these problems.

1.8.4. INSULIN EXPRESSION, BIOSYNTHESIS AND SECRETION

The islet β-cell is exquisitely sensitive to blood glucose levels, and rapidly adjusts insulin secretion accordingly, thus maintaining normoglycaemia at all times. The processes by which the β-cell achieves this degree of control are dependent upon complex, multi-faceted intracellular signalling systems, that are currently impossible to reproduce in a non-β-cell. However, many studies have attempted to identify the mechanisms involved in insulin production and secretion in the hope that this knowledge may be used to produce an alternative genetically-modified insulin-secreting cell.

The tissue-specific regulation of insulin gene expression is dependent on promoter and enhancer sequences located upstream of the transcription start site (785,786). Recently cDNA sequences of insulin gene regulatory proteins have been determined (787-789), one of which specifically binds to the most important transcriptionally-active promoter/enhancer sequence. Welsh et al (790) reported that the insulin gene responds to glucose over long periods of time, but until the glucose response elements within the insulin gene are further characterised, expression of the insulin gene in transfected cells is best controlled by constitutive viral promoters.

The transcription of the insulin gene generates a pre-mRNA which is processed in the nucleus. The mature preproinsulin mRNA is then transferred to the cytoplasm where it is translated. This process represents the predominant short-term control of insulin production in response to blood glucose (791). Insulin is then synthesised as its larger precursor preproinsulin, which undergoes post-translational proteolysis during its transfer through the cellular secretory apparatus (792,793). The prepeptide is removed by a signal peptidase located on the luminal surface of the endoplasmic reticulum. Within the endoplasmic reticulum, proinsulin folds to form the native disulphide bonds of insulin, and is transferred via a vesicular compartment to the Golgi apparatus. Proinsulin then leaves the golgi in immature granules which develop into insulin storage granules (794), before being processed to insulin by endopeptidase (795) and carboxypeptidase (796,797) action. Fibroblast and keratinocyte cells have no endogenous prohormone-processing endopeptidases, and therefore insulin gene transfected cells would secrete proinsulin rather than insulin.

The response of the β-cell to glucose is dependent on glucose metabolism within the cell rather than the transport of glucose into the cell, which is not a rate-limiting step. Evidence suggests that the enzyme glucokinase, which is involved in the first step in glucose metabolism, serves as a glucosensor linking the metabolism of glucose to the electrical and ionic changes associated with the secretory response of the β-cell. Both the β-cell transporter (Glut 2) (798) and glucokinase (799) have been cloned and sequenced.

1.8.5. PRELIMINARY STUDIES

Although it may be impossible to engineer these complex control mechanisms in a surrogate cell, it might be constructive to evaluate the clinical usefulness of what can

be achieved with gene therapy. In a preliminary experiment, Selden et al (800) implanted a clonal fibroblast cell line expressing the human preproinsulin gene into diabetic mice. A transient fall in blood glucose levels, which paralleled an increase in insulin levels, was observed. Furthermore, 24 days after transplantation of the transfected cells, normoglycaemia was restored in these animals. It should be noted that the level of expression of the preproinsulin cDNA depends on the strength of the promoter located upstream of the cDNA. Since there is no regulated secretory pathway present in these cells (801), the expressed product will be secreted from the cell as it is produced, at a level determined by the number of cells transplanted. Proinsulin is approximately 8% as active as insulin in maintaining blood glucose control in diabetic patients (802), and acts by suppressing glucose output from the liver, with little stimulation of glucose disposal (803). The basic transfected cell could therefore be improved by introducing a gene encoding a processing endopeptidase along with the preproinsulin cDNA, so that proinsulin can be cleaved to the more active insulin.

More recently, Stewart et al (804,805) transfected pituitary AtT20 cells by calcium phosphate co-precipitation with cDNA encoding human preproinsulin. This gene is driven by the zinc-sensitive metallothionein-1 promoter which generates an approximately 17-fold increase in insulin levels in response to zinc ions (806). The transfected clone, AtT20MtIns-1.4, constitutively released insulin at a rate of approximately 4 ng/10^6 cells/24 hours when cultured in medium containing 10 mmol/l glucose, and >80% was shown to be human insulin by high pressure liquid chromatography. However, increasing the glucose concentration of the medium did not lead to a consequent increase in insulin secretion from these cells. In initial studies (804), 2 x 10^6 transfected cells were implanted i.p. into athymic nude mice, and the release of insulin *in vivo* was evaluated using a specific human C-peptide

assay. Human C-peptide was detected in recipient plasma after implantation, but 3 weeks after induction of diabetes with STZ, these mice became severely hyperglycaemic, despite C-peptide concentrations of approximately 0.1 pmol/ml. These findings indicate that a greater number of cloned cells are necessary to prevent hyperglycaemia. In a subsequent study (803), 5 x 10^6 AtT20MtIns-1.4 cells were implanted either i.p. or subcutaneously into STZ-diabetic immunocompetent nude (nu/nu) mice. Plasma human C-peptide concentrations of >0.1 pmol/ml were achieved within one day. These concentrations declined rapidly after subcutaneous implantation, but more slowly after i.p. implantation. Addition of zinc sulphate (500 mg/l) to drinking water immediately and significantly increased human C-peptide to >10 and >17 pmol/ml in subcutaneously and i.p. implanted mice respectively. The response was only maintained in the latter group. Despite these circulating levels of insulin, plasma glucose was not consistently lowered by the implants, reflecting insulin resistance in this model. Upon removal of the AtT20MtIns-1.4 cells on day 30, the subcutaneous implants were found to be larger but almost entirely necrotic when compared with the i.p. implants, further demonstrating that this peritoneal cavity favours the functional performance of genetically engineered insulin secreting pituitary cells.

In conclusion, advances have been made towards using genetic and cellular engineering as a potential treatment for diabetes. Indeed, somatic cells capable of *in vivo* insulin secretion have been constructed. However, the secretion of insulin is low (insufficient to reverse STZ-diabetes in mice) and more importantly, not regulated by glucose. The implant therefore produces a continual background secretion of insulin at a level determined by the number of cells implanted. Regulation of insulin secretion in response to glucose is undoubtedly the most difficult problem to overcome, and will probably never be engineered into a surrogate cell. It is more

feasible to envisage the use of genetically manipulated insulin-secreting cells in conjunction with insulin injections to attain normal blood glucose control in the diabetic patient, with resultant effects on the complications arising from hyperglycaemia.

1.9. OVERALL CONCLUSIONS

Human IDDM is a heterogenous and multifactorial disorder. Genetic factors play a major role in disease susceptibility and interact with environmental factors to precipitate the disorder. Animal models of diabetes, although unable to fully replicate all aspects of the human condition, have proved invaluable in elucidating the sequence of events leading to the onset of IDDM. The BB rat more accurately reflects human IDDM as it spontaneously develops diabetes, and although this model shares many similar characteristics with the NOD mouse, there is no sex difference in the propensity to develop diabetes in the BB rat. For these reasons, the BB rat is the animal model of diabetes used in the studies described in this thesis. Despite improved understanding of the pathogenesis of the disease, no safe and effective method of preventing diabetes is currently available. Prevention studies have been hindered by the need to identify factor(s) initiating onset of IDDM. In addition, a method that accurately predicts diabetes-susceptible individuals in the general population is still not available. For these reasons, many groups have concentrated their efforts on searching for improved methods of insulin delivery. CIT achieves poor metabolic control, and close-to-normal blood glucose values are not achieved, leading to severe long-term microvascular complications including morphological changes in the eyes, kidneys and nerves. Sustained release insulin implants achieve near-normal blood glucose concentrations but cannot respond to daily fluctuations in blood glucose. In contrast, glucose sensors respond to changes in blood glucose

concentrations but do not function *in vivo* long-term due to their unreliability and bioincompatibility. An alternative approach to daily exogenous insulin administration as a treatment of IDDM is replacement of pancreatic islets destroyed by the autoimmune process. Transplantation of isolated islets has several advantages compared with vascularised pancreas grafts, although this procedure is not problem-free. Islets transplanted into an autoimmune model of IDDM are rapidly destroyed by recurrence of the autoimmune process. Immunosuppression, immunoalteration and induction of tolerance are three possible ways to circumvent these problems. Alternatively, bioartificial pancreases isolate islets from the hosts immune system by an artificial membrane, and include extravascular and vascular systems. Failure of these systems is generally due to bioincompatibility of the foreign material used to form the membrane. Somatic cell gene therapy might resolve the problems of tissue rejection and recurrent autoimmunity if target cells taken from the diabetic patient are used for the insertion of the insulin gene. However, the inability to regulate insulin secretion in these cells in response to glucose stimulation is an obstacle that will be extremely difficult to overcome in the genetically engineered surrogate cell. Thus, all the aforementioned systems designed to improve the delivery of insulin to the diabetic patient are not currently problem-free. Only progress towards resolving these problems will permit these systems to be successfully used in the clinical treatment of IDDM.

The studies described in this thesis comprise three novel approaches to improved insulin treatment of human IDDM using the insulin-dependent BB/E rat as an animal model of spontaneous autoimmune diabetes.

(i) Comparison of effect of treatment with daily single subcutaneous injections of insulin, i.e. CIT, and SRII on metabolic control and feeding patterns in BB/E and STZ-diabetic rats and comparison with non-diabetic rats.

(ii) Comparison of insulin secretory response of freshly isolated free islets and free and microencapsulated islets, cultured for different periods of time, using a perifusion system to determine whether a recovery culture period is necessary for optimal function of APA microencapsulated rat islets.

(iii) Determination of effect of short-term anti-CD4 and anti-CD8 moAb therapy on intraportal islet allograft survival in well-established diabetic BB/E rats.

CHAPTER 2

ACHIEVING AND ASSESSING METABOLIC CONTROL IN RATS WITH SPONTANEOUS INSULIN-DEPENDENT AUTOIMMUNE AND STREPTOZOTOCIN-INDUCED DIABETES : COMPARISON OF METABOLIC CONTROL AND FEEDING PATTERNS IN NON-DIABETIC AND DIABETIC RATS TREATED CONVENTIONALLY WITH SUBCUTANEOUS INJECTIONS OF INSULIN OR SUSTAINED RELEASE INSULIN IMPLANTS.

2.1. INTRODUCTION

The use of animal models for the study of the development of IDDM not only depends on the availability of an animal displaying a diabetic syndrome similar to human IDDM, but also on accurate methods of assessing glycaemic control. In addition, a means of achieving and maintaining various levels of metabolic control for prolonged periods are mandatory for studies involving the development of diabetic microangiopathy. Limited information is available on daily blood glucose profiles of diabetic BB rats treated by CIT, despite the importance of ensuring that a random blood sample taken for blood glucose determination is representative. Animals that are normoglycaemic several hours following a single injection of insulin may later return to a hyperglycaemic state, since insulin action does not continue throughout the day. This observation may be relevant to analysis of experimental data involving " normoglycaemic " BB rats.

In this study, the effects of CIT and SRII on metabolic control and feeding patterns in spontaneously diabetic BB/E and STZ-diabetic rats were compared with non-diabetic rats. Plasma glucose concentrations and food intake were measured every 2 hours for 24 hours and HbA_1 values were determined. In addition, tissue concentrations of the principal metabolites of the polyol pathway were determined in STZ-diabetic rats at the end of study.

2.2. MATERIALS AND METHODS

2.2.1. ANIMALS

2.2.1.1. The BB/E rat

The Edinburgh colony of inbred spontaneously diabetic, insulin-dependent BB rats (designated BB/E) was established in 1982 from animals donated by Dr. Pierre Thibert at the Animal Resources Division in Canada, Ottawa and is therefore derived from the original BB colony. The BB/E colony consists of two sublines of animals created by selective breeding. The DP mainline was established by selecting breeding pairs from high incidence litters, where at least 55% of the litter would develop diabetes. The incidence of diabetes in the DP-BB/E line is 50-60% with a mean age at onset of diabetes of 96 days. The DR subline was derived by selecting rats from litters of low incidence (<30%). If any of the mating pairs subsequently developed diabetes at any stage, they and their progeny were killed. The incidence of diabetes amongst the DR-BB/E rats is zero.

BB/E rats are maintained in rooms with independent heating and ventilation from the rest of the Biomedical Research Facility. Ventilation involves two sets of filters to produce 100% fresh air (21 $\pm$ 2^{o}C, 50% humidity). Animals are kept in an automatic light cycle of 12 hours light/dark. Animals are allowed free access to food (SDS rat and mouse no.1 Expanded Diet, Special Diet Services, Witham, UK) and water. All animals are weighed twice weekly to monitor for diabetes onset and indicate control of diabetes in rats receiving insulin. Diagnosis of diabetes is based on a continuous decrease in body weight and confirmed by the presence of glycosuria and hyperglycaemia. Glycosuria is detected using Multistix (Ames Division, Miles

Laboratories Ltd., Slough, UK) and values of ≥14 mmol/l are regarded as a positive result. Blood glucose is measured following tail vein sampling using ExacTech blood glucose test strips (MediSense Britain Ltd., Birmingham, UK) in conjunction with an ExacTech blood glucose sensor. Readings of ≥ 18 mmol/l are regarded as indicative of diabetes onset. Following diagnosis, BB/E rats are maintained on single daily subcutaneous injections of Ultralente MC insulin (40 I.U./ml) (Novo Nordisk, Copenhagen, Denmark) administered between 0900-1000 hours. The dose of insulin is adjusted for individual animals according to regular measurements of body weight and the degree of glycosuria.

2.2.1.2. The normal Wistar albino rat

Normal Wistar albino rats were bred and maintained in the Biomedical Research Facility, University of Edinburgh. Diabetes was chemically-induced in these animals by a single i.v. injection of 65 mg/kg streptozotocin (Sigma Chemical Co. Ltd., Poole, Dorset, UK) administered in citrate buffer, pH 4.5 (2.1 g citric acid [BDH Chemicals Ltd., Poole, Dorset, UK], 20 ml 1 M NaOH made up to 100 ml with sterile distilled water) into the tail. Diabetes was confirmed by subsequent blood glucose concentrations of >18 mmol/l, weight loss and glycosuria.

2.2.2. INSULIN TREATMENT

2.2.2.1. Conventional insulin therapy

BB/E rats were initially maintained on a single daily subcutaneous injection of 2.4 - 4.0 I.U. of insulin. CIT ceased upon implantation of Linplant SRII (Møllegaard, Skensved, Denmark)

2.2.2.2. Sustained release insulin implants

SRII are made by high pressure compression of a powder admixture of 17% bovine insulin and 83% recrystallised palmitic acid. Entrapped insulin is gradually released from the implant (7 mm long with a diameter of 2 mm) by slow surface erosion *in vivo*. For this reason, even when the implant is broken into smaller pieces, there is little change in the insulin release rate, although less insulin will be released per day. The insulin release rate from a single SRII is approximately 2 units/day for ar least 40 days. The dosage varies with age of the animal and severity of its diabetes. The recommended dosage for an adult BB rat with mild to severe diabetes is 1 or 2 implants. If given an optimal implant dose, glycosuria and ketonuria are absent in the recipient since a set basal dose of insulin is continuously released during the day. In contrast, glycosuria and ketonuria are difficult to prevent using CIT since the action of the injected insulin does not last throughout the day.

2.2.2.2.1. Insertion of sustained release insulin implants

Recommended sites of subcutaneous insertion of an SRII include upper abdominal and dorsal skin and neck region. Animals receiving an implant were anaesthetised with halothane and the site of injection shaved and cleaned with Betadine antiseptic solution (Napp Laboratories, Cambridge, UK). The skin was pinched and pierced with a 16G needle and a 12G trocar (Møllegaard) was pushed through the skin orifice created following brief immersion in 2% Betadine solution. The implant was briefly immersed in 2% Betadine solution prior to insertion into the proximal end of the trocar and the implant was pushed to the distal end with an obturator until it exited the trocar. The skin was pinched over the inserted implant and the trocar withdrawn. The orifice required no suturing or clips for closure. Progressive erosion

of the SRII starts immediately following implantation, and the effect of the released insulin on blood glucose level can be detected in less than 1 hour.

Since preliminary experiments indicated that diabetic BB/E rats implanted with 2 SRII died of hypoglycaemia (blood glucose concentration <2 mmol/l), rats were implanted with a single SRII in the majority of subsequent studies.

2.2.3. EXPERIMENTAL PROTOCOL

2.2.3.1. Metabolic control in BB/E rats

Four groups of BB/E rats were studied : (1) non-diabetic BB/E rats (n = 13) comprising both DR-BB/E (n = 9) and DP-BB/E (n = 4) rats; (2) diabetic DP-BB/E rats treated by CIT (n = 19); (3) diabetic DP-BB/E rats treated by a single SRII implanted subcutaneously in the sternal region (n = 13) and (4) diabetic DP-BB/E rats initially treated by a half-sized piece of SRII, i.e. 3.5 mm long and 2 mm diameter, implanted subcutaneously in the back neck region with the subsequent implantation of 0-4 quarter-size pieces, i.e 1.8 mm long and 2 mm diameter (mean SRII = 1¼, range = ½-1½), so as to maintain normoglycaemia (n = 6). The mean (± SEM) duration of diabetes prior to implantation of SRII was 137 ± 11 days. Blood glucose concentration and body weight were measured three-times each week after SRII implantation (daily for group 4). Urine samples were also monitored for the presence of glucose and ketones. All diabetic BB/E rats were subjected to a 24 hour plasma glucose analysis 7 days before substituting CIT with SRII and again 27 days after implantation of SRII to assess the variation in 24 hour glycaemic control. Plasma glucose concentrations of non-diabetic rats were also determined over a 24 hour period. Blood samples were collected for plasma

glucose estimations every 2 hours from 0800 to 0800 the following day. The initial blood sample taken at 0800 was also used to determine HbA_1.

2.2.3.2. Metabolic control in STZ-diabetic rats

Four groups of rats were studied : (1) normal Wistar rats (n = 4); (2) untreated STZ-diabetic Wistar rats (n = 6); (3) STZ-diabetic Wistar rats treated by CIT (n = 15) and (4) STZ-diabetic Wistar rats treated by a single SRII implanted subcutaneously in the sternal region (n = 10). Untreated STZ-diabetic rats were diabetic for 8 days prior to determination of 24 hour plasma glucose concentrations and treated STZ-diabetic rats were diabetic for 12 ± 1 days before starting CIT or SRII. 24 hour plasma glucose profiles were assessed 28 ± 1 days later.

2.2.3.3. Feeding study

Food intake was measured every 2 hours over a 24 hour period in the following 7 groups of rats : (1) non-diabetic Wistar rats (n = 4); (2) non-diabetic BB/E rats (n = 13) comprising both DR-BB/E (n = 9) and DP-BB/E (n = 4) rats; (3) diabetic DP-BB/E rats treated by CIT (n = 6); (4) diabetic DP-BB/E rats treated by variable amounts of SRII (mean = 1¼, range ½-1½) implanted subcutaneously in the back neck region (n = 6); (5) untreated STZ-diabetic Wistar rats (n = 6); (6) STZ-diabetic Wistar rats treated by CIT (n = 15) and (7) STZ-diabetic Wistar rats treated by a single SRII implanted subcutaneously in the sternal region (n = 10). The rats were given a known weight of food at the start of the study and the quantity eaten every 2 hours determined. Animals were allowed free access to food and water.

2.2.4. GLYCATED HAEMOGLOBIN ANALYSIS

HbA_1 values were determined by the method described by Tames et al (807). Briefly, mixed venous/arterial blood samples (approximately 100 µl) were collected from the tails of non-anaesthetised rats and mixed (50 units/ml of blood) with preservative-free heparin (Multiparin, CP Pharmaceuticals Ltd., Wrexham, UK) on parafilm strips and transferred to a 1.5 ml Sarstedt tube (Sarstedt Ltd., Leicester, UK). Blood was centrifuged (3500 x g, 3 minutes) and plasma removed for subsequent glucose estimation using a Beckman Glucose Analyser 2 (Beckman Instruments [UK] Ltd., High Wycombe, Buckinghamshire, UK). Red blood cells were washed three times in 0.9% saline and incubated overnight with an equal volume of saline at room temperature. The cells were centrifuged and the packed cells haemolysed by addition of 3 volumes of a 1 : 10 dilution of haemolysing reagent containing 0.1 % saponin and 0.05% ethylenediaminetetra-acetic acid (EDTA) in distilled water (Corning Medical and Scientific Ltd., Halstead, UK). After vortex mixing for 15 seconds, the cells were centrifuged (3500 x g, 5 minutes) and 1 µl of the haemolysate containing approximately 40 µg of rat haemoglobin applied to individual sample wells on citrate agar plates (Corning).

Rat haemoglobin variants were seperated by electroendosmosis (Corning " Glytrac " electrophoresis system, 1.5 x 17 cm band width) using 0.1 mmol/l citrate buffer (pH 6.3) under 60 V for 40 minutes. The percentage of HbA_1 was determined by automatic scanning densitometry on a Corning 720 fluorometer/densitometer equipped with a 420 nm filter.

2.2.5. TISSUE POLYOL ANALYSIS

Tissue concentrations of sorbitol, fructose, glucose and *myo*-inositol were determined in nerve, kidney and lens samples of insulin-treated STZ-diabetic rats at the end of study by capillary gas chromotography of trimethylsilyl ether derivatives (808) by Dr R M Lindsay. In brief, α-methylmannoside (500 nmol) was added as internal standard to approximately 50-100 mg of tissue in polypropylene tubes and boiled for 25 minutes. After cooling, proteins were precipitated by sequential addition of zinc sulphate (200 µl; 5%) and barium hydroxide (200 µl; 5%). After centrifugation (1000 x g, 15 minutes), the supernatant was transferred to glass tubes and lyophilised. Samples were derivatised for 24 hours by adding 300 µl Tri-Sil reagent (Pierce and Warriner [UK] Ltd., Chester, UK) then extracted by adding 2 ml distilled water and 300 µl cyclohexane. After centrifugation (1000 x g, 15 minutes), the cyclohexane layer was removed and 1 µl injected into a Hewlett-Packard HP5890 series II gas chromatograph (Hewlett-Packard Ltd., Cheadle Heath, Cheshire, UK). Quantitation was achieved using a Hewlett-Packard HP3396A integrator by comparison with authentic mixed standards of fructose, glucose, sorbitol and *myo*-inositol containing α-methylmannoside as internal standard extracted simultaneously.

2.2.6. STATISTICAL ANALYSIS

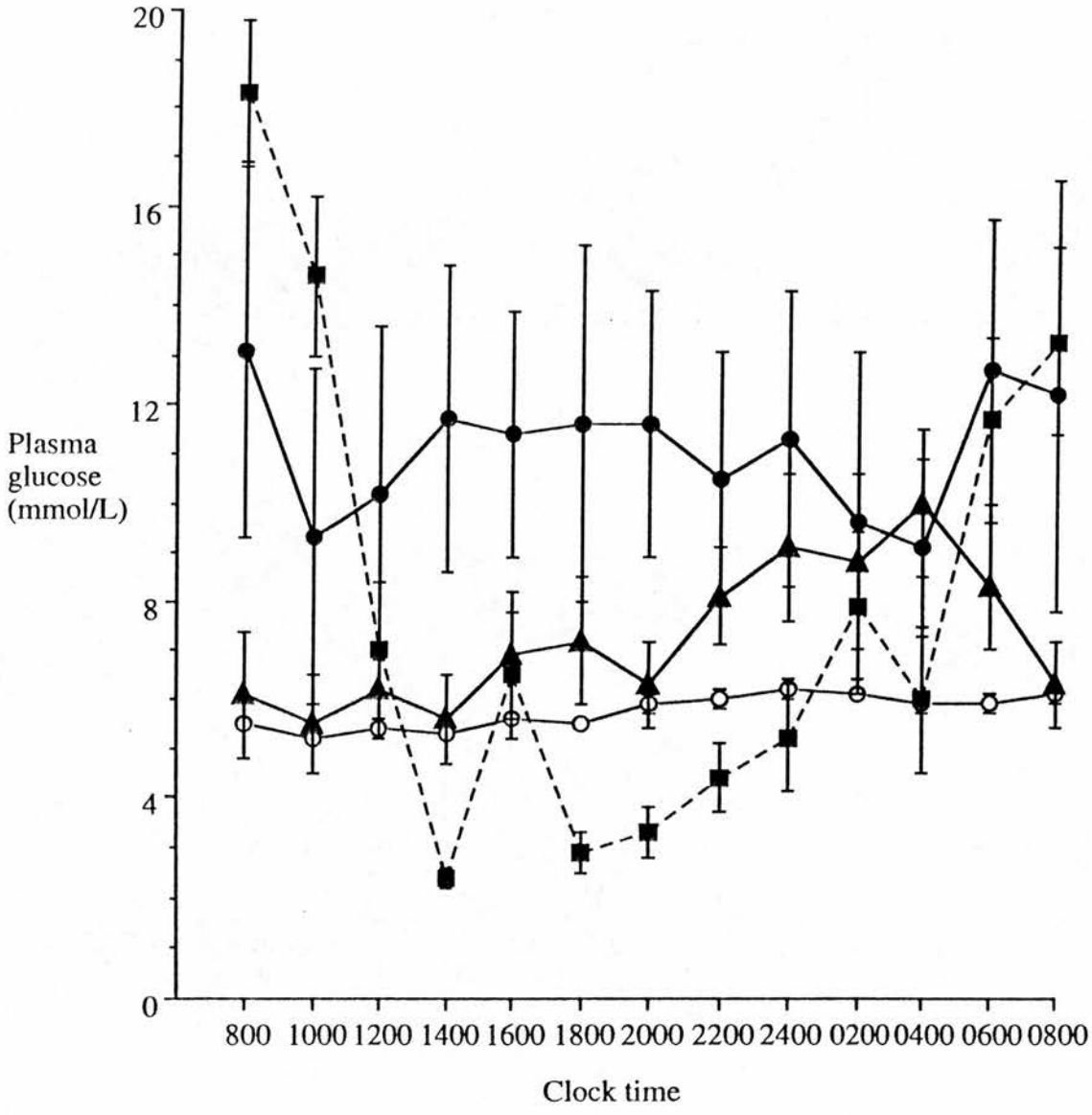
All results are presented as mean ± standard error of the mean (SEM) and were analysed using unpaired Student's t-test. Regression equations and scatter plots were determined by a least squares procedure using a Minitab program (CLECOM Ltd., Birmingham, UK).

2.3. RESULTS

Figure 1 shows the 24 hour plasma glucose profiles of CIT-treated diabetic BB/E rats 7 days prior to transfer to SRII treatment and subsequently 27 days later. Mean plasma glucose concentration of single SRII-treated (7.3 ± 0.4 mmol/l, range = 5.5 - 10.0 mmol/l) or variable (mean = 1¼) SRII-treated (11.1 ± 0.5 mmol/l, range = 9.1 - 13.1 mmol/l) but not CIT-treated (8.0 ± 1.4 mmol/l, range = 2.4 - 18.3 mmol/l) diabetic BB/E rats were significantly higher ($p<0.001$) than non-diabetic rats (mean = 5.7 ± 0.1 mmol/l, range = 5.2 - 6.2 mmol/l). Diabetic BB/E rats initially treated with a half-sized piece of SRII and subsequently quarter-sized pieces (mean = 1¼) were slightly hyperglycaemic and the mean plasma glucose concentration was significantly higher than that of single SRII-treated BB/E rats ($p<0.001$). Mean plasma glucose concentration of CIT-treated BB/E rats was not significantly different from diabetic BB/E rats treated with a single SRII, but was significantly lower ($p<0.05$) than animals treated with variable amounts of SRII. Diabetic BB/E rats maintained by CIT showed considerable diurnal variation in plasma glucose levels whereas both SRII treatments reduced these fluctuations. The service life, i.e. the length of sustained action, of a single SRII was 55 ± 4 days which was significantly higher ($p<0.001$) than that of variable amounts of SRII (31 ± 4 days).

Mean HbA_1 values in diabetic BB/E rats treated with CIT or a single SRII were identical (4.9 ± 0.4% and 4.9 ± 0.2 % respectively) despite the greater diurnal variation and higher mean plasma glucose concentration observed in the CIT-treated group. Mean HbA_1 of diabetic rats treated with variable amounts of SRII (6.2 ± 0.5%) was significantly higher ($p<0.05$) than diabetic BB/E rats treated with a single SRII but not CIT. BB/E rats treated with either a single or variable amounts of

Figure 1. 24 hour plasma glucose profiles in diabetic BB/E rats maintained by CIT or SRII.



Mean ± SEM

Plasma glucose concentrations were measured over a 24 hour period in non-diabetic (o , n=13) and diabetic BB/E rats initially treated by CIT (■ , n=19) and subsequently treated with either a single SRII (▲, n=13) or variable (mean = 11/4, range = 1/2 - 11/2) amounts of SRII (● , n=6). Non-diabetic rats comprised of both DR-BB/E (n=9) and DP-BB/E (n=4) rats.

Profiles of CIT-treated rats were performed 7 days prior to transfer to SRII treatment. Profiles of SRII-treated rats were performed 27 days after inititiation of SRII treatment.

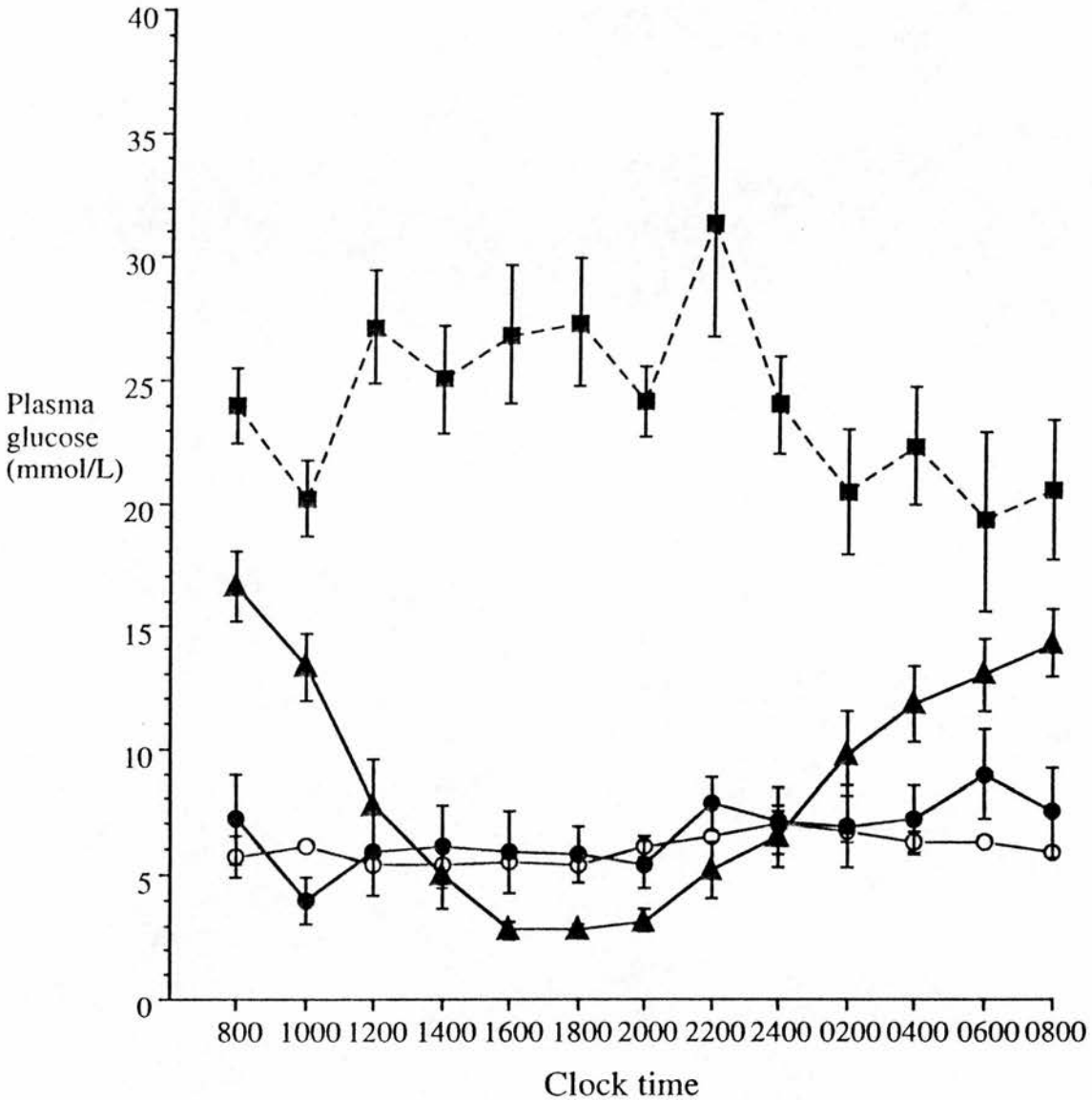
SRII, but not CIT, had mean HbA_1 values that were significantly higher ($p<0.05$ and $p<0.01$ respectively) than non-diabetic BB/E rats (4.2 ± 0.2%). Mean values of plasma glucose concentration and HbA_1 of DP-BB/E and DR-BB/E rats which comprised the non-diabetic group were not significantly different.

The 24 hour plasma glucose profiles performed on STZ-diabetic rats maintained by CIT or SRII are shown in Figure 2. The mean plasma glucose concentration (24.1 ± 1.0 mmol/l, range = 19.3 - 31.4 mmol/l) of untreated STZ-diabetic rats was significantly higher than CIT-treated (8.6 ± 1.3 mmol/l, range = 2.8 - 16.6 mmol/l) and SRII-treated (6.6 ± 0.4 mmol/l, range = 4.0 - 9.0 mmol/l) STZ-diabetic rats and non-diabetic (6.0 ± 0.1 mmol/l, range = 5.4 - 7.0 mmol/l) Wistar rats ($p<0.001$). Both CIT and SRII treatment lowered the mean plasma glucose concentration in STZ-diabetic rats and these values were not significantly different from the mean plasma glucose concentrations observed in non-diabetic Wistar rats. Treatment of STZ-diabetic rats with a single SRII reduced the diurnal fluctuations observed in CIT-treated rats.

CIT- and SRII-treated STZ-diabetic rats had lower HbA_1 values (mean = 7.4 ± 1.0% and 6.2 ± 0.6% respectively, $p<0.01$) compared with untreated STZ-diabetic Wistar rats (mean = 9.3 ± 0.8%). CIT- and SRII-treated diabetic rats both had significantly higher mean HbA_1 values compared with non-diabetic (4.4 ± 0.4%) rats ($p<0.05$).

Figure 3 shows the correlation between mean plasma glucose concentration and HbA_1 in (a) spontaneously diabetic BB/E rats and (b) STZ-diabetic rats treated with either CIT or SRII. The correlation between these two parameters

Figure 2. 24 hour plasma glucose profiles in STZ-diabetic BB/E rats maintained by CIT or SRII.

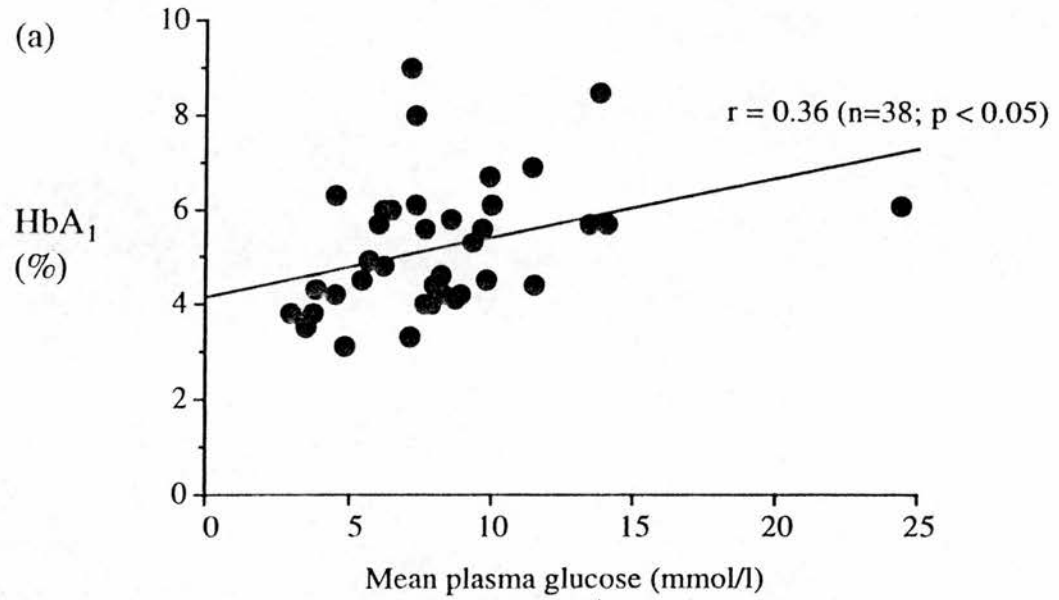


Mean ± SEM

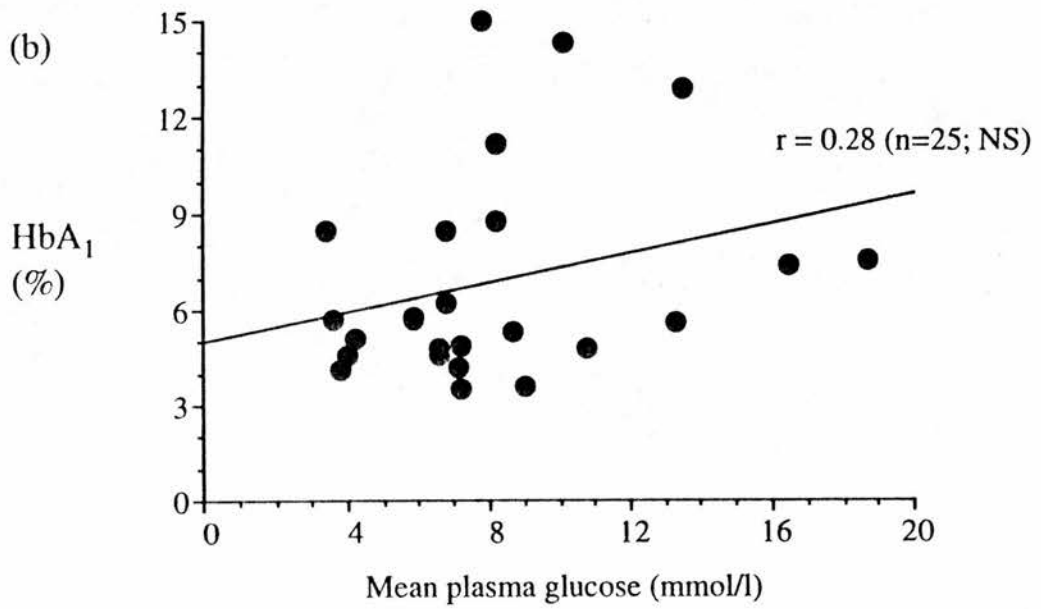
Plasma glucose concentrations were measured over a 24 hour period in non-diabetic (○, n=4) and untreated (■, n=6), CIT-treated (▲, n=6) and SRII-treated (●, n=6) STZ-diabetic rats.

Plasma glucose profile of untreated STZ-diabetic rats was performed 8 days after confirmation of diabetes. Insulin treatment of diabetic rats was initiated 12±1 days after confirmation of diabetes and plasma glucose profiles performed 28 days later. SRII-treated rats received a single SRII.

Figure 3. Correlation between HbA_1 and mean plasma glucose concentration in (a) spontaneously-diabetic BB/E and (b) STZ-diabetic rats.



Diabetic BB/E rats (n=19) were inititially treated with CIT and subsequently treated with a single (n=13) or variable (mean = 11/4) amount (n=6) of SRII.



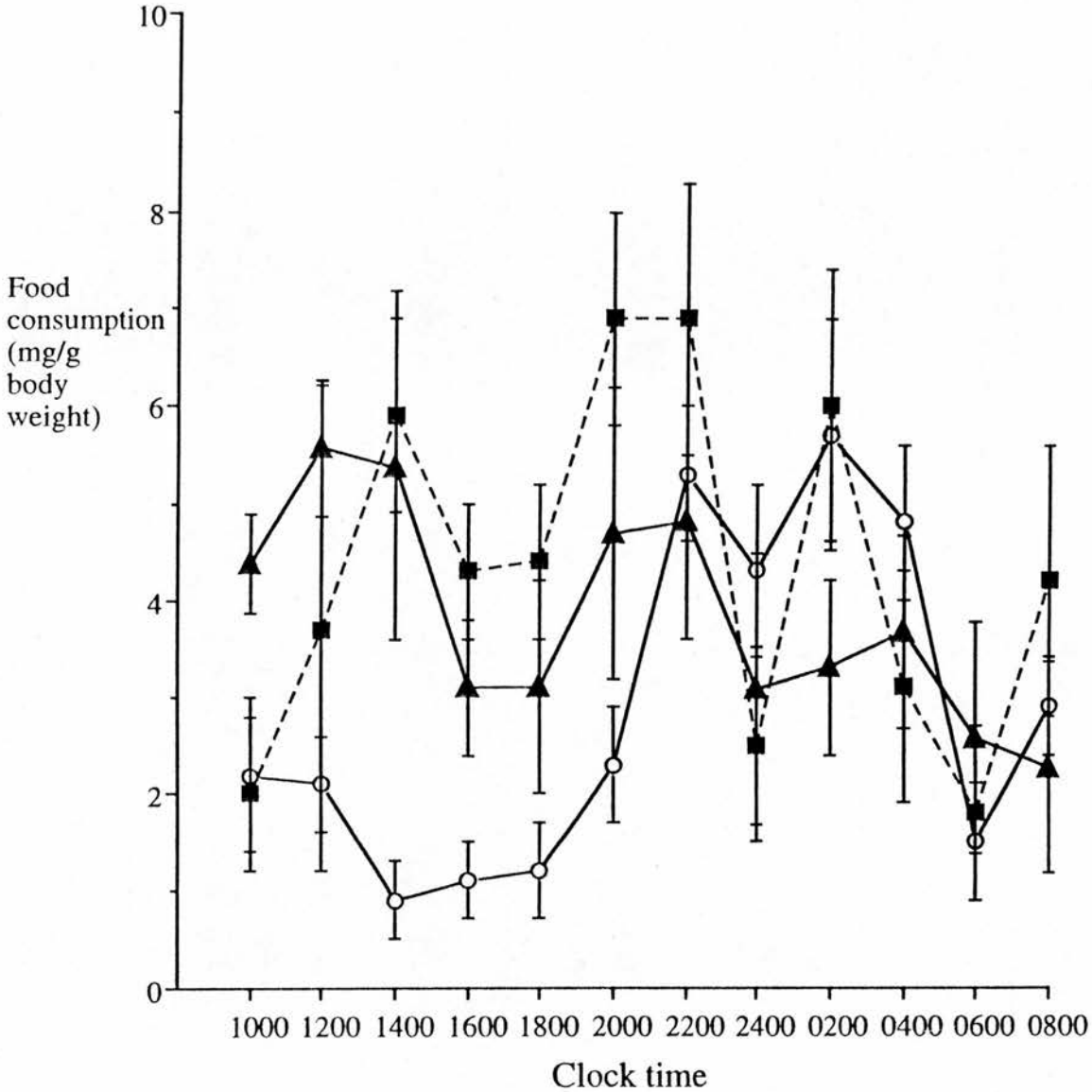
STZ-diabetic rats were treated with either CIT (n=15) or an single SRII (n=10).

was significant for spontaneously diabetic BB/E rats ($r = 0.36$, $p<0.05$) but not for STZ-diabetic rats ($r = 0.28$).

Twenty-four hour feeding profiles in non-diabetic and diabetic BB/E rats maintained by CIT or variable amounts (mean = 1¼) of SRII are shown in Figure 4. Non-diabetic rats comprised DP-BB/E and DR-BB/E rats and there was no significant difference between the amount of food consumed during either the 12 hour light and dark cycles or the 24 hour period of study between these two groups. Non-diabetic BB/E rats consumed significantly more food during the dark cycle than the light cycle ($p<0.001$). Diabetic BB/E rats treated with CIT or SRII did not consume significantly different amounts of food during the light and dark cycle and ate significantly more during the light cycle compared with non-diabetic BB/E rats ($p<0.001$). Food intake of CIT-treated diabetic and non-diabetic rats during the dark cycle was not significantly different. However, SRII-treated rats ate significantly less food compared with CIT-treated diabetic and non-diabetic rats ($p<0.05$). The total amount of food consumed in a 24 hour period by CIT- and SRII-treated diabetic BB/E rats was not significantly different, although CIT-treated (but not SRII-treated) animals consumed significantly more food than non-diabetic rats in the 24 hour period of study ($p<0.01$).

Figure 5 shows the 24 hour feeding profiles in non-diabetic and STZ-diabetic Wistar rats either untreated or maintained by CIT or a single SRII subcutaneously implanted in the sternal region. Non-diabetic Wistar rats ate significantly more food during the dark cycle than the light cycle ($p<0.001$). Untreated and SRII-treated rats did not eat significantly different amounts of food during the light and dark cycle and CIT-treated rats ate significantly more food during the light cycle than the dark cycle ($p<0.01$). All diabetic animals (untreated, CIT- and SRII-treated) ate significantly

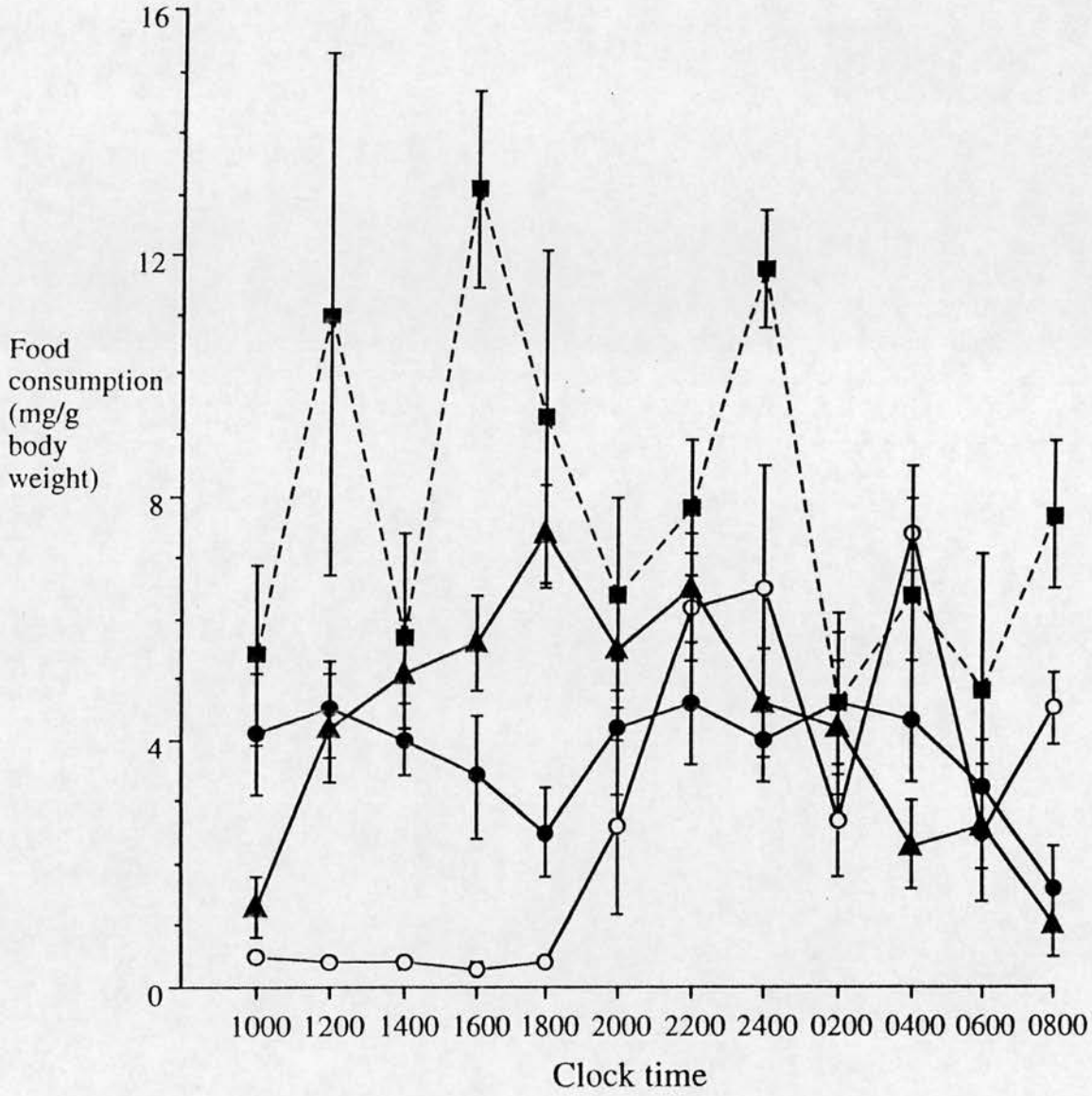
Figure 4. 24 hour feeding profiles in diabetic BB/E rats maintained by CIT or SRII.



Mean ± SEM

Food consumption was measured over a 24 hour period in non-diabetic (o , n=13) and diabetic BB/E rats treated with CIT (■ , n=6) and subsequently after 27 days treatement with variable (mean = 11/4, range 1/2 - 11/2) amounts of SRII (▲ , n=6). Non-diabetic rats comprised of both DR-BB/E (n=9) and DP-BB/E (n=4) rats.

Figure 5. 24 hour feeding profiles in STZ-diabetic Wistar rats maintained by CIT or SRII.



Mean ± SEM

Food consumption was measured over a 24 hour period in non-diabetic (o , n=4) and untreated (■ , n=6), CIT-treated (▲ , n=6) and SRII-treated (● , n=6) STZ-diabetic rats. SRII-treated rats received a single SRII.

more during the light cycle compared with non-diabetic rats ($p<0.001$). Untreated STZ-diabetic rats consumed significantly larger amounts of food than CIT- and SRII-treated rats during both the light ($p<0.05$ and $p<0.01$ respectively) and dark ($p<0.01$) cycles and also ate significantly more than non-diabetic Wistar rats ($p<0.05$) during the dark cycle. However, non-diabetic Wistar rats consumed significantly greater amounts of food than both CIT- and SRII-treated rats during the dark cycle ($p<0.05$). There was no significant difference in food consumption of CIT-treated and SRII-treated rats during either the light or dark cycles. The total amount of food consumed over the 24 hour period of study by non-diabetic Wistar rats was significantly less than the food consumed by untreated and CIT-treated (but not SRII-treated) rats ($p<0.01$ and $p<0.001$ respectively). Untreated STZ-diabetic rats consumed significantly more food than CIT- and SRII-treated diabetic rats ($p<0.01$), and CIT-treated rats ate more food than SRII-treated and non-diabetic rats.

Table 1 compares the food consumption and metabolic control in non-diabetic and diabetic rats. Non-diabetic BB/E rats consumed significantly more food than non-diabetic Wistar rats during the light cycle ($p<0.05$). When the data for non-diabetic rats was combined and compared with that for all diabetic rats, the latter group were found to consume significantly more food during the 12 hour dark cycle and the 24 hour period of study and had significantly higher mean plasma glucose and HbA_1 values ($p<0.001$).

The results of analysis of the principal metabolites of the polyol pathway in sciatic nerve, kidney and lens samples from CIT- and SRII-treated STZ-diabetic rats are shown in Table 2. Data obtained from previous studies in the Metabolic Unit for non-diabetic and untreated STZ-diabetic rats is also presented in Table 2 for

Table 1. Comparison of food consumption and metabolic control in non-diabetic and diabetic rats

Rat Strain	Insulin treatment	n	FOOD CONSUMPTION (mg/g body weight) 12 hour light cycle	12 hour dark cycle	24 hour food consumption	24 hour plasma glucose concentration (mmol/l)	HbA_1 (%)
Non-diabetic Wistar	-	4	4.6 ± 1.1^{c}	$29.7 \pm 2.0^{a,d,f}$	$34.3 \pm 2.7^{b,g}$	6.0 ± 0.1^{c}	4.4 ± 0.4^{c}
Non-diabetic BB/E	-	13	9.8 ± 1.5^{k}	$24.5 \pm 1.6^{d,e}$	34.3 ± 2.8	5.7 ± 0.1	4.2 ± 0.2
All non-diabetic	-	17	8.6 ± 1.2	25.7 ± 1.4^{d}	34.3 ± 2.1	5.8 ± 0.1	4.2 ± 0.2
Spontaneously diabetic BB/E	CIT	6	25.5 ± 2.9^{j}	24.3 ± 1.3^{e}	49.8 ± 3.2^{i}	8.5 ± 1.4	5.2 ± 0.3^{h}
	SRII	6	21.9 ± 1.9^{j}	19.7 ± 1.5	41.6 ± 3.0	11.1 ± 0.5^{j}	6.2 ± 0.5^{i}
STZ-diabetic Wistar	-	6	50.8 ± 8.1^{l}	43.0 ± 4.7	93.8 ± 12.6	24.1 ± 1.0	9.3 ± 0.8
	CIT	15	$29.3 \pm 1.6^{a,l}$	22.7 ± 1.7^{c}	52.0 ± 2.1^{b}	8.6 ± 1.3^{c}	7.4 ± 1.0^{k}
	SRII	10	$22.6 \pm 2.8^{b,l}$	21.7 ± 2.7^{b}	44.3 ± 5.0^{b}	6.6 ± 0.4^{c}	$6.2 \pm 0.6^{b,k}$
All diabetic		43	29.5 ± 2.0^{m}	25.1 ± 1.5	54.6 ± 3.3^{m}	10.4 ± 1.0^{m}	7.5 ± 0.4^{m}

Mean ± SEM

[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ compared with untreated STZ-diabetic rats.
[d] $p<0.001$ compared with 12 hour light cycle.
[e] $p<0.05$ compared with SRII-treated BB/E rats.
[f] $p<0.05$ compared with CIT- and SRII-treated STZ-diabetic rats.
[g] $p<0.001$ compared with CIT-treated STZ-diabetic rats.
[h] $p<0.05$, [i] $p<0.01$ and [j] $p<0.001$ compared with non-diabetic BB/E rats.
[k] $p<0.05$, [l] $p<0.001$ compared with non-diabetic Wistar rats.
[m] $p<0.001$ compared with all non-diabetic rats.

Table 2. Effect of CIT and SRII treatment on tissue polyol pathway activity in STZ-diabetic rats.

		Non-diabetic	STZ-diabetic		
Insulin treatment		-	-	CIT	SRII
n		10	6	9	11
Tissue	Metabolite (nmol/g tissue)				
Sciatic nerve	glucose	802 ± 124	5777 ± 672*	394 ± 77***	518 ± 165
	sorbitol	278 ± 28‡	2975 ± 300*	107 ± 31*	46 ± 10
	fructose	668 ± 49	4804 ± 177*	1111 ± 323	562 ± 110
	myo-inositol	3523 ± 341††	2346 ± 151	3384 ± 468	2851 ± 238
Kidney	glucose	3316 ± 63†,‡	10425 ± 837*	1255 ± 183	1676 ± 360
	sorbitol	142 ± 14	624 ± 112*	174 ± 52	200 ± 48
	fructose	409 ± 9	604 ± 35*	618 ± 80***	556 ± 73
	myo-inositol	3916 ± 190†††	3103 ± 224	5069 ± 234**	5403 ± 192*
Lens	glucose	69 ± 5‡‡	2005 ± 214*	458 ± 106**	246 ± 69
	sorbitol	278 ± 18	22659 ± 2737*	898 ± 155*	501 ± 189
	fructose	238 ± 16	8404 ± 491*	1563 ± 294*	1220 ± 332**
	myo-inositol	1136 ± 55†	137 ± 11	1263 ± 151	1033 ± 140

Mean ± SEM

*** $p<0.05$, *$p<0.01$ and *$p<0.001$ compared with non-diabetic rats.
††† $p< 0.05$, †† $p< 0.01$ and † $p< 0.001$ compared with untreated diabetic rats.
‡‡ $p<0.05$ and ‡ $p< 0.001$ compared with SRII-treated rats.

comparison. Tissue levels of glucose, sorbitol and fructose are significantly increased and *myo*-inositol concentrations significantly reduced in untreated diabetic rats as previously reported (809). Both CIT and SRII treatment normalised tissue concentrations of these metabolites although the differences between the two insulin therapies were not significant.

2.4. DISCUSSION

The sustained delivery of insulin by an implant is thought to achieve improved physiological blood glucose control than CIT. Initially Brown et al (533) used non-degradable ethylene-vinyl acetate copolymer matrices to entrap insulin and a single device controlled plasma glucose concentration for up to 105 days in STZ-diabetic rats. However, removal of the implant was necessary once the insulin supply was exhausted. Other problems with long-term use include toxicity and lack of biocompatibility. Use of natural, non-antigenic materials may resolve these problems if the insulin delivery is comparable to that of versatile synthetic polymers. Water insoluble lipids have been studied since they are natural constituents of all tissues. Cholesterol is a suitable matrix for insulin delivery but the observed poor biodegradability *in vivo*, even after one year (534), is clinically undesirable in IDDM patients. In a subsequent study of 11 lipids, Wang (810) reported that palmitic acid (a long chain fatty acid) containing 20% insulin had the longest service life (37-50 days) and achieved near-normoglycaemic blood glucose profiles. Palmitic acid implants were also gradually eroded *in vivo* with a half-life of approximately 3 months in the rat and no fibrous overgrowth was observed on these SRII.

In this study, the degree of metabolic control achieved in spontaneously diabetic BB/E and STZ-diabetic rats by different insulin treatment regimens was assessed. For this

purpose, plasma glucose concentration and HbA_1 (determined using the electrophoretic method described by Tames et al [807]) were measured. This method determines HbA_1 without interference from either Hb variants or labile glycated fractions, the importance of which has been described in IDDM patients (811). Elevated HbA_1 values have previously been reported in both genetically diabetic mice (812,813) and rats (807) and in mice (813) and rats (814,815) with chemically-induced diabetes. This study also demonstrated increased HbA_1 values in diabetic BB/E and STZ-diabetic rats compared with non-diabetic rats. Furthermore, HbA_1 values observed in CIT-treated diabetic BB/E rats were significantly lower ($p<0.05$) than those reported for STZ-diabetic animals as previously reported (814, 815).

Two different methods of insulin treatment were evaluated in BB/E and STZ-diabetic rats : CIT and SRII. Both insulin regimens were effective in lowering the raised plasma glucose levels associated with IDDM. However, two SRII implanted subcutaneously in the sternal region of two diabetic BB/E rats resulted in fatal hypoglycaemia after 4 and 20 days. Diabetic BB/E rats initially maintained by half a single SRII in the back neck region (the site of CIT administration) followed by 0-4 quarter-sized pieces (mean = 1¼) were difficult to control and were slightly hyperglycaemic. This observation was reflected by significantly higher mean plasma glucose concentration and HbA_1 compared with BB/E rats maintained on a single SRII implanted in the sternal region. A single SRII reduced fluctuations in plasma glucose concentrations of CIT-treated diabetic BB/E and STZ-diabetic rats during the 24 hour period as reflected by lower values of SEM and range of plasma glucose concentration in SRII-treated diabetic rats. However, mean plasma glucose concentration of SRII-treated diabetic rats was higher than non-diabetic animals and significantly so for BB/E rats. In addition, the sequential implantation of variable

amounts of SRII to achieve normoglycaemia was less convenient. The service life of variable amounts of SRII implanted in the back neck region was also significantly shorter than a single SRII implanted in the sternal area. Several explanations for this difference include the simultaneous and increased erosion of the multiple pieces of SRII implanted in the former group due to increased exposure of surface area, and weakening of the implant pieces following dissection. In this study, implantation site also appears to play a role in determining the length of service life although no information is currently available to confirm this preliminary finding.

Although mean HbA_1 values of CIT- and SRII-treated rats were not significantly different, only SRII-treatment significantly decreased mean HbA_1 value compared with untreated STZ-diabetic rats. Mean HbA_1 values of CIT- and SRII-treated rats were significantly higher than non-diabetic rats indicating that neither insulin treatment achieved long-term normoglycaemia. The mean HbA_1 value of SRII-treated diabetic BB/E rats was also significantly higher than non-diabetic rats. There was no significant relationship between mean plasma glucose concentration and HbA1 in insulin-treated STZ-diabetic rats which is in agreement with a previous report (816). However, the correlation between these two parameters just reached significance for diabetic BB/E rats in contrast to the results of Tames et al (807).

Mean HbA_1 and plasma glucose concentrations of CIT- and SRII-treated diabetic BB/E rats were not significantly different despite the more pronounced glycaemic excursions observed in the former group. These apparently conflicting results may be explained by considering the 24 hour plasma glucose profiles. The considerable diurnal variation in plasma glucose concentration in CIT-treated spontaneously diabetic and STZ-diabetic rats meant these animals were only briefly normoglycaemic. Between approximately 0600 - 1000 hours, CIT-treated rats were hyperglycaemic

whereas following administration of insulin at 1000 hours, plasma glucose levels rapidly decreased to hypoglycaemic levels between 1400 - 2200 hours. This marked fluctuation in the 24 hour plasma glucose profile has previously been reported in CIT-treated diabetic rats (807). The near-normal mean plasma glucose and HbA_1 values in spontaneously diabetic and STZ-diabetic rats maintained on CIT indicate that metabolic control cannot be adequately assessed by either a single random measurement of plasma glucose concentration or HbA_1. For this reason, plasma glucose concentration should be determined at both 0800 hours (i.e. pre-CIT) and 1400 hours when CIT-treated animals are hyperglycaemic and hypoglycaemic respectively. In contrast, measurement of a single random plasma glucose concentration or HbA_1 accurately reflects the metabolic control of diabetic rats maintained on SRII. Tames et al (807) reported similar results after studying long-term glycaemic control achieved in diabetic BB/E rats maintained by continuous insulin infusion using an osmotic minipump, or CIT. However, in contrast to the surgical implantation and limited service life (14 days) of osmotic minipumps, SRII are implanted simply with minimal surgery and no special aftercare, and maintain good glycaemic control for 55 ± 4 days.

Since SRII cannot respond to varying insulin demands, Wang (535) determined the extent of blood glucose fluctuation that might result in dangerous convulsions due to feeding schedules. Wang observed reasonably stable blood glucose levels of STZ-diabetic Wistar rats implanted with a single SRII under self-feeding or scheduled feeding conditions. Animals also tolerated an overnight (16 hour) fast and hypoglycaemic convulsion was not observed despite blood glucose levels of approximately 2.5 mmol/l.

In this study, diabetic rats ate significantly more food during the 12 hour light cycle and over the 24 hour period of study compared with non-diabetic rats. This was primarily due to the fact that diabetic rats fed continuously throughout the 24 hour day irrespective of glycaemic control and insulin treatment. In contrast, non-diabetic rats consumed significantly more food during the dark cycle, when these animals are active, compared with the light cycle. Indeed, non-diabetic BB/E and Wistar rats ate significantly more food during the dark cycle than SRII-treated diabetic BB/E rats and CIT- and SRII-treated STZ-diabetic rats respectively. Interestingly, non-diabetic BB/E rats ate significantly more food than non-diabetic Wistar rats during the light cycle. Diabetic BB/E rats treated with CIT or SRII ate similar amounts of food although CIT-treated rats consumed significantly more food during the dark cycle. Untreated STZ-diabetic rats were most severely hyperglycaemic and hyperphagic. Insulin treatment with CIT or SRII lowered mean plasma glucose concentrations to near-normal values, and HbA_1 values and food consumption decreased accordingly suggesting that hyperphagia in severely diabetic animals may be reduced but not normalised by insulin treatment. The feeding patterns observed in BB/E or STZ-diabetic rats may be explained when considering the role of neuropeptide Y (NPY), a potent central appetite stimulant. NPY is a 36 amino acid neurotransmitter related to pancreatic polypeptide (817) and found at high concentrations in the hypothalamus where it is synthesized in neurons in the arcuate nucleus (ARC) (818). These neurons, together with specific neurons in the medulla, principally project to the paraventricular nucleus (PVN) and dorsomedial nucleus (DMN), both of which are major appetite-regulating areas within the hypothalamus (819). Upon injection into PVN, DMN and other hypothalamic sites, NPY induces carbohydrate-selective hyperphagia (820) even at extremely low dosages. Indeed, the effect is so intense and sustained that repeated administration induces weight gain and obesity (820). These findings suggest that NPY plays an important role in

controlling food intake and body weight *in vivo*. In fasted rodents, NPY is increased in the PVN and to a lesser extent in other hypothalamic nuclei (821) and NPY mRNA is increased in the ARC (822). Increased hypothalamic NPY concentrations have also been reported in insulin-deficient BB/E and STZ-diabetic rats (823,824) and the increases in STZ-diabetic rats were subsequently localised to specific hypothalamic regions (ARC, PVN and DMN) implicated in metabolic and neuroendocrine regulation (825). Increased NPY concentrations, together with raised NPY mRNA content in the ARC, downregulation of NPY receptor numbers in the hypothalamus and enhanced NPY release within the PVN (823-827) suggest that synthesis, transport and release of NPY in the hypothalamus are increased in diabetic rats. In view of the documented central action of NPY in stimulating appetite, this peptide is thought to mediate the hyperphagia characteristic of untreated diabetes.

McKibbin et al (828) reported that insulin deficiency rather than hyperglycaemia was the major stimulus for elevation of hypothalamic NPY in STZ-diabetic rats and that hyperglycaemia may exert an inhibitory effect on feeding. Insulin crosses the blood-brain barrier from the circulation and interacts with insulin receptors on neurons in the ARC where it acts as a satiety signal, serving to regulate body weight (829). Indeed, peripheral insulin administration to diabetic rats reduces hypothalamic NPY to near-basal levels (830) and normalises food intake (828). Furthermore, Schwartz et al (831) recently reported that insulin may inhibit NPY gene expression directly. In agreement with these results, reduced hyperphagia was observed in insulin-treated STZ-diabetic rats as shown previously (830, 832). Metabolic control of SRII-treated rats was more stable compared with CIT treatment and this was reflected by the lower food consumption of these rats. If indeed NPY mRNA in the ARC responds relatively rapidly to small changes in circulating insulin as suggested by Marks et al (833), a consequent decrease in NPY concentration in

SRII-treated diabetic rats may explain the greater reduction of food consumed by these animals compared with CIT-treated diabetic rats. However, although insulin treatment reduced food consumption in diabetic animals, hyperphagia was not abolished in spontaneously diabetic and STZ-diabetic rats treated by CIT or SRII as both groups ate significantly more than non-diabetic BB/E and Wistar rats. This finding is in agreement with Williams et al (823) who reported that even in intensively insulin-treated BB/E rats, physiological blood glucose values were not achieved and central hypothalamic NPY levels were (as in poorly controlled groups) significantly higher than in non-diabetic DR-BB/E rats.

This study also investigated the effect of CIT and SRII treatment on polyol pathway activity in three tissues susceptible to development of diabetic microangiopathy. Excessive glucose flux through the polyol pathway to produce elevated tissue levels of sorbitol and fructose and the associated reduction in tissue *myo*-inositol concentration have been implicated in the pathogenesis of diabetic vascular complications (194, 195). CIT and SRII treatments both corrected the characteristic abnormalities in tissue levels of these metabolites observed in untreated STZ-diabetic rats. This result contrasts the significantly higher values of mean glucose concentration and HbA_1 observed in both groups of insulin-treated STZ-diabetic rats. One factor which may account fro this difference is the time at which tissues were sampled since polyol pathway activity closely parallels blood glucose concentration. Tissue samples were collected between 1100 to 1200 hours when plasma glucose levels were decreasing following recent insulin administration in CIT-treated rats. This time point occurs at approximate normoglycaemia in these animals (7.0 ± 1.4) and would suggest similar correction of tissue polyol pathway metabolism. The insignificant differences in concentration of the principal polyol pathway metabolites between CIT- and SRII-treated groups may also be due to the similar plasma glucose

concentrations at this time point in these two groops. However, sampling of tissues at other time points may have revealed differences in polyol pathway activity in CIT- and SRII-treated rats which could be important in the development of diabetic complications.

In summary, this study demonstrates that a single SRII implanted subcutaneously in the sternal region can achieve and maintain relative normoglycaemia in spontaneously diabetic BB/E and STZ-diabetic rats and lower food consumption towards that of non-diabetic rats. Different degrees of glycaemic control can be achieved by varying the number of SRII implanted, and the metabolic status of the recipient is accurately reflected by random assessment of plasma glucose concentration and HbA_1 thus providing an alternative to large numbers of plasma glucose determinations. In addition, the daily plasma glucose fluctuations observed in diabetic rats maintained on CIT, which may contribute to long-term complications related to the diabetic condition, are eliminated in SRII-treated rats. Indeed, sorbitol concentrations were reduced in the sciatic nerve, kidney and lens of STZ-diabetic rats treated with SRII, although this reduction was not significantly different to the decreases observed in diabetic rats maintained by CIT.

It will be difficult to estimate the number of SRII required to clinically treat human IDDM patients using rat body weight and implant size. Further studies with diabetic animals of larger sizes are necessary to supplement results obtained using rats. However, SRII do not respond to glucose fluctuations and only provide a sustained basal daily dosage of insulin. Since insulin demand varies with meal intake and physical activity, supplemental doses of insulin in addition to the basal dose of insulin provided by SRII, will be necessary. Wang (834) recently demonstrated that the transient hyperglycaemia observed in alloxan-diabetic rabbits maintained on a single

SRII (containing 15% insulin) after drinking sweetened water could be interrupted by a supplemental bolus of insulin provided by a silicone implant. This second implant contained compressed insulin, some of which was dissolved when serous fluid entered the internal volume of the implant through an orifice. When required, sideways compression of the silicone implant over the abdominal skin fold of the rabbit delivered the supplemental dose. Further research is required to determine whether this open-loop arrangement can be modified to a closed-loop system in the IDDM patient by using SRII in conjunction with an implant with internal modulation capability to provide the additional doses of insulin to counteract postprandial hyperglycaemia.

CHAPTER 3

THE IMPORTANCE OF A RECOVERY CULTURE PERIOD FOR OPTIMAL FUNCTION OF ALGINATE - POLY-L-LYSINE - ALGINATE MICROENCAPSULATED RAT ISLETS.

3.1. INTRODUCTION

Microencapsulation of islets of Langerhans in an APA membrane is a potentially effective method to prevent allograft and xenograft rejection without the need for immunosuppression. However, the application of the microencapsulation technique to islet transplantation has had variable success. Although the maintenance of an adequate insulin response by encapsulated islets is a prerequisite for successful transplantation, few studies have determined glucose-induced insulin secretion of APA encapsulated islets. Furthermore, conflicting results have been reported upon testing the capacity of APA microencapsulated islets to secrete insulin in response to glucose challenge *in vitro*. Lim and Sun (738) and Darquy and Reach (736) reported that microencapsulated islets responded adequately to glucose stimulation, whereas Chicheportiche and Reach (744) and Fritschy et al (743) observed very poor glucose-induced insulin response by islets after microencapsulation.

The aim of this study was to investigate the glucose-induced insulin secretory response of APA microencapsulated islets and to determine whether the encapsulation procedure had any effect on islet function and the effect of a period of culture post-encapsulation. Microencapsulated islet function was compared with the insulin secretion of freshly isolated and cultured free islets.

3.2. MATERIALS AND METHODS.

3.2.1. ANIMALS

3.2.1.1. The normal Wistar albino rat

Normal Wistar rats were bred and maintained in the Biomedical Research Facility, University of Edinburgh.

3.2.2. ISOLATION OF PANCREATIC ISLETS

Pancreatic islets were isolated from normal male Wistar albino rats (200-250 g body weight) by two different collagenase digestion methods depending on whether the islets were to be cultured or used immediately after isolation, i.e. fresh.

3.2.2.1. Isolation of islets for culture

Animals were anaesthetised throughout the procedure using fluothane inhalation anaesthetic (Zeneca Ltd., Cheshire, UK). The abdomen was opened by a midline incision to expose the pancreas and the pancreatic duct and the distal duct was clamped off at its point of attachment to the duodenum. The proximal duct was cannulated with a Portex cannula (diameter 3FG) (Portex Ltd., Hythe, Kent, UK). Collagenase type XI (Sigma) was diluted to 0.43 mg/ml in Hanks Buffered Salt Solution (HBSS) (Sigma) containing 5.5 mmol/l glucose and supplemented with 0.04% sodium bicarbonate (Imperial Laboratories Ltd., Andover, Hants, UK), 0.1% BSA (Sigma), 20 mmol/l Hepes (Imperial Laboratories Ltd.), 100 U/ml penicillin, 0.1 mg/ml streptomycin (Imperial Laboratories Ltd.) and 7.5 mmol/l calcium

chloride (Sigma), and placed on ice. The pancreas was inflated by injecting 6 ml of the collagenase solution through the cannula over a 30 second period. The inflated pancreas was carefully dissected from the attached gut tissue and placed on ice in a sterile universal container until sufficient pancreases were obtained. The digestion reaction was started upon the addition of 5 ml of prewarmed (37°C) supplemented HBSS (Ca^{2+}-free) containing 4% BSA to each pancreas, and continued in a static water bath at 37°C for exactly 16½ minutes. The reaction was stopped by pouring the digest into 20 ml of cold (4°C) HBSS medium containing 0.1% BSA, and the suspension was vigourously hand-shaken for 60 seconds. Digests were centrifuged (173 x g, 2 minutes) at 4°C and the supernatants discarded. The tissue pellet was resuspended in 15 ml of cold HBSS medium and further disaggregated by drawing and expelling the suspension 7 times in a 20 ml syringe with a sterile 14G steel needle. The digest was then passed through two sterile 1 mm pore size steel meshes to remove fat and ductal tissue. The filtrate was centrifuged as before and after decanting the supernatants, the digests were separated from contaminating exocrine elements by layering onto a discontinuous dextran gradient.

Dextran T70 (Pharmacia, Uppsala, Sweden) was diluted in supplemented HBSS medium to the following densities : 1.105, 1.095, 1.070 and 1.060 g/ml and used to create a four-layer density gradient. Gradients were centrifuged (500 x g, 25 minutes) at 20°C and the islets, once removed from between the first and second interfaces, were washed in 50 ml of supplemented HBSS, followed by a second wash in 15 ml of HBSS. Islets were further purified by hand picking under a stereomicroscope.

Seperated islets were washed twice in 15 ml of HBSS and once in RPMI 1640 medium (Sigma) containing 11.1 mmol/l glucose and supplemented with

0.2% sodium bicarbonate, 20 mmol/l Hepes, 2 mmol/l L-glutamine (Imperial Laboratories Ltd.), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Islets were cultured free-floating in sterile Falcon tissue culture dishes (Becton Dickinson, Cowley, Oxford, UK) containing 5 ml of RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (FCS) (Imperial Laboratories Ltd.) in a fully humidified atmosphere of 5% CO_2 at 37°C. Each culture dish contained 400-500 islets, and media was changed 24 hours after islet isolation and every 2 days thereafter.

3.2.2.2. Isolation of fresh islets

Animals were killed by cervical dislocation and an immediate laparotomy was performed. The pancreas was located and dissected out as previously described into a tissue culture dish containing CO_2-free salt solution with the following ionic composition : Na^+ 141 mmol/l; K^+ 5.9 mmol/l; Ca^{2+} 2.5 mmol/l; Mg^{2+} 1.2 mmol/l; PO_4^{3-} 1.2 mmol/l and Cl^- 101 mmol/l (BDH); pH 7.4. The medium was supplemented with the sodium salts of glutamic, lactic and fumaric acids (Sigma) at a concentration of 5 mmol/l, 0.55% BSA fraction V (Pentex, Miles Inc. Diagnostics Division, Illinois, USA) and 2.8 mmol/l glucose. After removal of excess fat the pancreas was inflated with 10 ml of cold (4°C) CO_2-free salt solution using a 25G needle (Microlance, Becton Dickinson, Dublin, Ireland) and finely minced. The tissue was washed twice with CO_2-free salt solution and twice with bicarbonate-buffered salt solution containing 24.9 mmol/l HCO^-_3 (BDH) in addition to the CO_2-free salt solution, and was continually gassed (5% CO_2 and 95% O_2) to maintain pH 7.4, and transferred to a 25 ml conical flask. Collagenase type XI was diluted to 5 mg/ml in bicarbonate-buffered salt solution and added to the minced pancreas, and incubated in a shaking water bath at 37°C for 8-9 minutes, with

vigorous 30 second hand-shaking every minute. Upon completion of the collagenase incubation, the suspension was transferred to a universal container and washed three times with CO_2-free salt solution and three times with bicarbonate-buffered salt solution. In between washes, the islets were allowed to settle under gravity for 90 seconds before the supernatant was removed. After the last wash, islets were resuspended in 5 mls of bicarbonate-buffered salt solution and transferred to a siliconised black tissue culture dish and hand-picked under a dissecting microscope. Islets, which appeared as free, round or ovoid structures with a greyish-white colour, were pooled in fresh bicarbonate-buffered salt solution and immediately transferred to the perifusion apparatus.

3.2.3. MICROENCAPSULATION OF ISLETS

Islets were cultured for 48 hours prior to microencapsulation by a method originally described by Lim and Sun (738) and modified by O'Shea et al (740). Islets were suspended in a sterile-filtered solution of 1.5% (w/v) sodium alginate (Sigma) at a concentration of approximately 2000/ml. The suspension was transferred to a 10 ml syringe containing a small magnet and connected to the droplet-forming apparatus as described by Ennis and James (835). The flow rate of the syringe pump (Razel model A-99, Razel Scientific Instruments Inc, Stamford, Conneticut, USA) was set at 0.66 ml/min and the air flow at approximately 2000 ml/minute. Islet-containing droplets were extruded through a stainless steel 22G needle surrounded by a purpose built air jacket and collected in a large tissue culture dish containing 50 ml of 100 mmol/l calcium chloride (BDH). The distance from the tip of the needle to the surface of the calcium chloride solution was exactly 4 cm, and upon contact with the calcium chloride solution the islet-containing alginate droplets formed solid beads which gravitate to the bottom of the tissue culture dish. At the end of the extrusion,

the gel beads were transferred to a sterile 50 ml Corning centrifuge tube (Corning Incorp., New York, USA) and the supernatant decanted down to 15 ml. The gel beads were washed three times with sterile 0.9% saline (Baxter Healthcare Ltd., Norfolk, UK) and the volume reduced to 5 ml. The gel beads were then treated with 30 ml of 0.1% 2-N-(cyclohexamino)-ethane-sulphonic acid (CHES) buffer, pH 8.2 (Sigma) for 3 minutes on a rotator and washed, prior to coating of the beads in 30 ml of 0.05% poly-L-lysine (molecular weight by viscosity : 21 500; Sigma) for 10 minutes. Capsules were immediately treated with 30 ml of 0.1% CHES for 3 minutes, washed twice and finally coated in 30 ml of 0.15% sodium alginate (Sigma) for 4 minutes. The solid inner core of the capsules was reliquified by rotation in 10 ml of 55 mmol/l sodium citrate, pH 7.4 (Sigma) for 6 minutes, and after two final saline washes the microcapsules were incubated in supplemented RPMI-1640 at 37°C in a fully humidified atmosphere of 5% CO_2. Under a low power stereomicroscope, the microcapsules were approximately 600 μm in diameter, and possessed a perfectly smooth and spherical outer membrane. Only microcapsules containing a single islet were used in this study.

3.2.4. PERIFUSION OF ISLETS

The perifusion chambers consisted of a 3.5 cm length of 100 μl Eppendorf pipette tips (Eppendorf, Hamburg, Germany), the narrow end of which was occluded with a piece of sponge on which the islets were placed. Bicarbonate-buffered salt solution containing either 2.8 mmol/l (non-stimulatory) or 16.6 mmol/l (stimulatory) glucose was continually gassed (5% CO_2 and 95% O_2). The glucose concentrations of the media were checked using Beckman Synchron Clinical System CX3 (Beckman Instruments [UK] Ltd.). Non-stimulatory medium was pumped over the islets at a rate of approximately 1 ml/minute using a multichannel Desaga PLG peristaltic pump.

This instrument produced a minimal pulsatile flow and hence minimised physical agitation of the islets. A three-way tap placed between the pump and the media resevoirs allowed the selection of either non-stimulatory or stimulatory medium. Silicone tubing with an internal diameter of 0.5 mm connected the perifusion chamber with both the three-way taps and the LP3 collecting tubes (Denley Instruments Ltd., West Sussex, UK). The total dead space was approximately 0.5 ml. The perifusion chambers, media reservoirs and as much of the interconnecting tubing as possible were immersed in a thermostatically controlled water bath at 37^{o}C.

Twelve chambers could be perifused simultaneously and each channel was loaded with 30 representative islets selected from a common pool. In this study, islets were (a) freshly isolated (n = 18 channels), (b) cultured for either 4 (n = 5 channels), 6, 9 or 13 days (n = 7 channels each), or (c) encapsulated after 2 days culture and cultured for a further 2, 4, 7 or 11 days (n = 7 channels each). The time interval between the removal of the pancreas and the administration of the first glucose stimulus was approximately 2 hours for freshly isolated islets. After loading, all islets were perifused for a minimum of 30 minutes with non-stimulatory medium. Islets were then subjected to two consecutive sustained glucose challenges with stimulatory bicarbonate-buffered medium, seperated by a 20 minute wash with non-stimulatory medium. Samples of perifusate were collected over 2 minute periods when islets were perifused with non-stimulatory medium. Upon switching to stimulatory medium, samples were collected at 1 minute intervals for the initial 10 minutes and over 2 minute intervals thereafter. After perifusion, samples were frozen at -20^{o}C prior to RIA for insulin content. Islets were washed with non-stimulatory medium followed by 0.9% saline for a minimum of 30 minutes, and the microcapsules examined under a light microscope to ensure structural integrity had been maintained throughout perifusion. The constancy of flow rate in each channel was checked during the course

of the experiment and any variation in flow rate between channels was accounted for when insulin secretory rates were calculated.

3.2.5. ANALYTICAL METHODS

3.2.5.1. Total insulin content

Washed islets were removed and suspended in 0.5 ml of distilled water and sonicated (Sonicator model A180G, Ultrasonics Ltd., Shipley, Yorkshire, UK) at speed and tuning setting 5 for 30 seconds (40 seconds for encapsulated islets). The insulin present in 100 µl of sonicate was extracted overnight in 900 µl of acidified absolute alcohol (Hayman Ltd., Witham, Essex, UK) at 4°C and total insulin content (TIC) determined by RIA. Insulin secretion by islets in each channel was standardised for TIC.

3.2.5.2. Insulin radioimmunoassay

Insulin was assayed by radioimmunoassay using the method described by Ashby and Speake (836). 100 µg of lyophilised rat insulin (Novo Nordisk) was reconstituted in 1 ml of distilled water and used to prepare a standard curve. This stock solution was diluted to 10 ng/ml with buffer B, containing 40 mmol/l sodium di-hydrogen orthophosphate (BDH), 0.6 mmol/l thiomersal (BDH), 0.5% BSA fraction V, 150 mmol/l sodium chloride and 25 µl/ml of trasylol (Bayer, Newbury, Berkshire, UK), pH 7.4, and 7 insulin concentrations (0.2 - 6 ng/ml) prepared. 100 µl of each standard and sample were pipetted in triplicate and duplicate respectively into LP3 tubes on ice and 200 µl of anti-porcine insulin guinea pig serum (Scottish Antibody Production Unit, Carluke, Lanarkshire, UK) diluted 1 : 15 000 in buffer A containing

40 mmol/l sodium di-hydrogen orthophosphate, 0.6 mmol/l thiomersal and 0.5% BSA fraction V, pH 7.4, added. Tubes were vortexed briefly and incubated for 2 hours at 4°C. After incubation, 100 µl (0.125 µCi/ml) of I^{125}-labelled insulin (Amersham, Aylesbury, UK) was added and tubes further incubated for 2 hours at 4°C. Following the second incubation, 400 µl of charcoal reagent (50 mg/ml Norit GSX charcoal [BDH] made up in heat-inactivated filtered horse serum [Imperial Laboratories Ltd.] diluted 1 : 2 with buffer A) was added, vortexed and allowed to stand for 10 minutes at room temperature. Following centrifugation (1300 x g, 30 minutes) at 4°C, 400 µl was diluted with 200 µl distilled water and transferred into duplicate LP3 tubes using a LKB 2075 diluter (BCL, Lewes, UK). Samples were counted for 60 seconds using an NE1600 gamma scintillation counter (Nuclear Enterprises, Edinburgh, UK). Values were expressed as a percentage of total count (100 µl sample of I^{125}-labelled insulin) and a standard curve of percentage total count against insulin concentration (ng/ml) constructed. Quality control samples of known insulin concentration were simultaneously analysed.

3.2.6. HISTOLOGY

Microencapsulated islets were fixed in neutral-buffered formalin (Gurr microscope materials, BDH; pH 7.2) after 7 days of culture. Immunohistochemistry was performed by Mr Lawrence Brett and sections analysed by Dr M McIntyre of the Department of Pathology, Western General Hospital, Edinburgh. Sections (4 µm thick) were stained with haematoxylin and eosin. Further sections were mounted on poly-L-lysine coated slides and immunostained for insulin and glucagon using a biotin-Streptavidin alkaline phosphatase conjugate method. Non-specific staining was blocked with 20% sheep serum in Tris-buffered saline for 10 minutes prior to incubation of sections in rabbit anti-insulin or rabbit anti-glucagon primary

antibody (Euro-path Ltd., Bude, Devon, UK) diluted 1 : 100 in a solution of 1% BSA in Tris-buffered saline for 30 minutes. After a Tris-buffered saline wash, primary antibodies were detected with a biotinylated $F(ab)_2$ antibody fraction (Boehringer Mannheim UK, Lewes, Sussex, UK) diluted 1 : 400 for 30 minutes. Following a further Tris-buffered saline wash, sections were incubated in Streptavidin/alkaline phosphatase conjugate (Boehringer Mannheim UK) diluted 1 : 1000 for 30 minutes. After a final Tris-buffered saline wash the alkaline phosphatase label was detected using New Fuchsin Chromogen (Dako Ltd., High Wycombe, Buckinghamshire, UK). Finally, sections were counterstained with haematoxylin, dehydrated and mounted in Synthetic Xylene Substitute Mountant (Life Sciences International, Basingstoke, Hampshire, UK). All incubations were performed at room temperature (approximately $20^{o}C$).

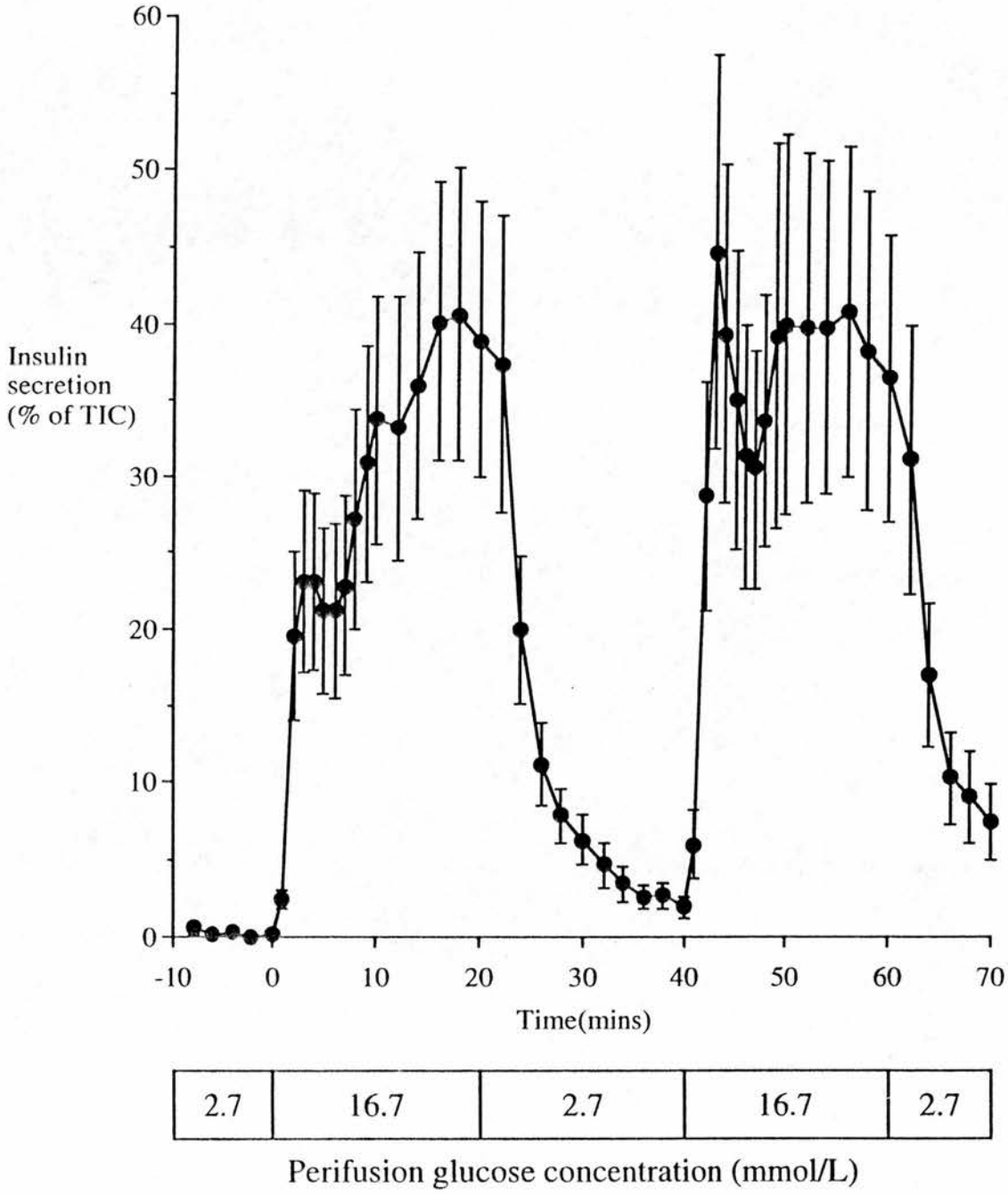
3.2.7. STATISTICAL ANALYSIS

All results are presented as mean $\pm$ SEM and were analysed by unpaired Student's t-test.

3.3. RESULTS

Figure 6 shows the insulin release pattern observed when freshly isolated rat islets were subjected to two sustained periods of stimulation with perifusion medium containing 16.7 mmol/l glucose. Eighteen channels (30 islets/channel) were perifused. Islets were initially equilibriated in perifusion medium containing a basal glucose concentration of 2.7 mmol/l. Upon switching to stimulatory glucose, a vigourous biphasic insulin secretory response was observed with clearly defined first and second phase insulin secretion. Total insulin secretion (% of TIC) expressed as

Figure 6. Insulin release pattern of freshly isolated rat islets during perifusion.



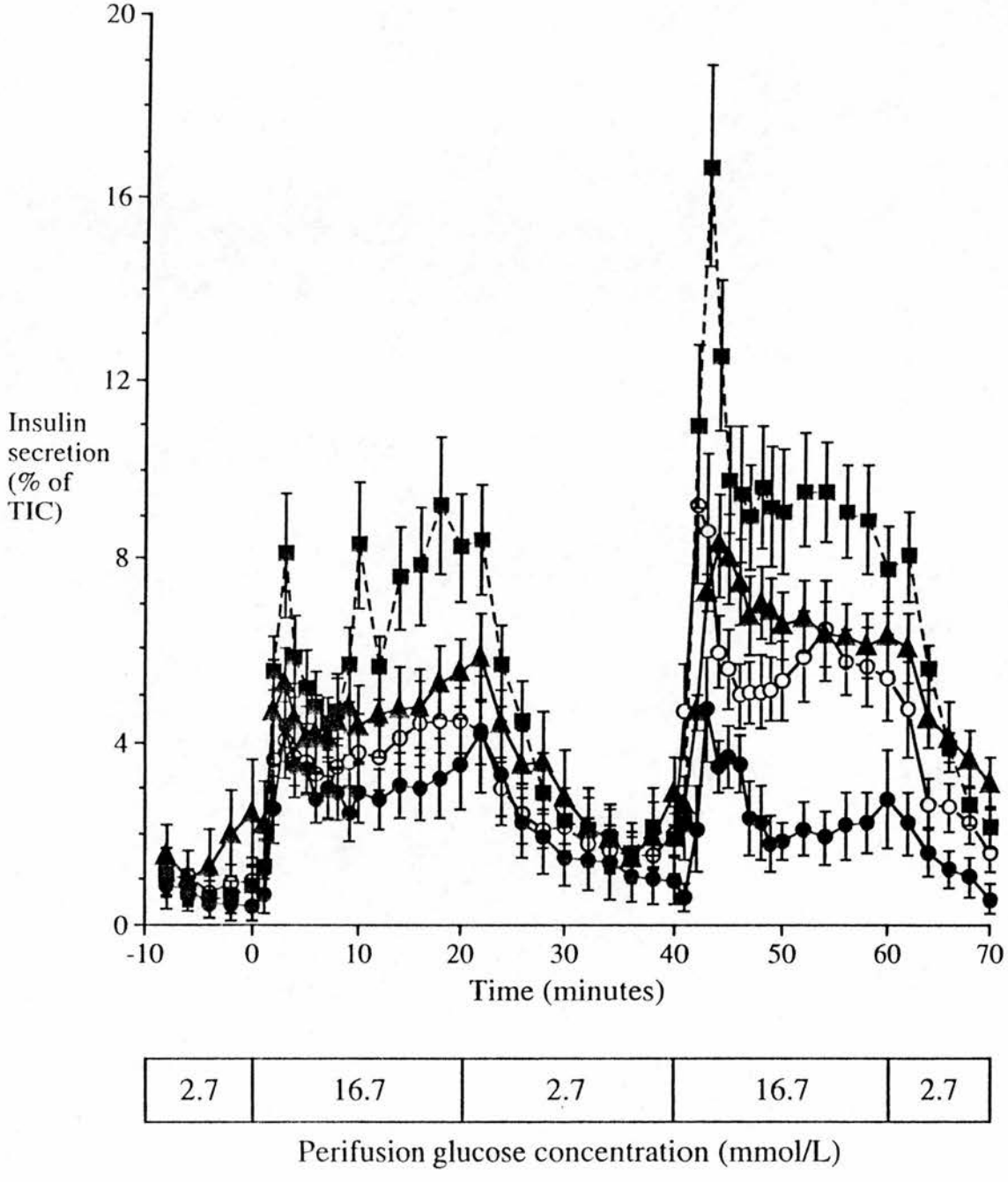
Mean ± SEM

18 channels (30 islets/channel) were perifused.

area under the curve (AUC) or peak first phase first stimulation was 589.0 ± 143.3% and 23.1 ± 6.0% respectively. Upon removal of stimulatory glucose medium, insulin release rapidly declined towards basal secretory levels. Following a 20 minute " rest " period in basal glucose medium, islets were subjected to a second high glucose stimulation. A more pronounced biphasic response was observed and the insulin secretion (% of TIC) was 699.8 ± 196.2% and 44.7 ± 13.0% when expressed as AUC or peak first phase second stimulation respectively.

The effect of culture period on insulin release pattern of free (non-encapsulated) rat islets during perifusion is shown in Figure 7. The biphasic insulin secretory profiles were qualitatively similar to those observed upon perifusion of freshly isolated islets. However, quantitatively, free cultured islets secreted significantly decreased amounts of insulin (% of TIC) at each culture period studied when compared with freshly isolated islets, whether insulin secretion was expressed as AUC or peak first phase following first and second glucose stimulation or as total AUC (i.e. sum of first stimulation AUC and second stimulation AUC). Free islets showed a maximal secretion of insulin on culture day 6 whether expressed as AUC or peak first phase following first and second glucose stimulation or as total AUC. Insulin secretion (% of TIC) expressed as AUC following first glucose stimulation decreased after the optimal culture period and this reached significance ($p<0.05$) on day 13. Following a second glucose stimulation, insulin secretion increased significantly from culture day 4 to the optimal culture period on day 6 ($p<0.05$) after which insulin secretion decreased and this was significant by day 13 ($p<0.001$). Insulin secretion on culture day 13 was significantly lower than insulin secretion on day 4 ($p<0.05$). Similar observations were made when considering insulin secretion expressed as total AUC. Insulin secretion increased significantly from culture day 4 to day 6 ($p<0.05$) and decreased thereafter, reaching significance on day 13 ($p<0.01$). Insulin secretion

Figure 7. The effect of culture period on insulin release pattern of free (non-encapsulated) rat islets during perifusion.



Mean ± SEM

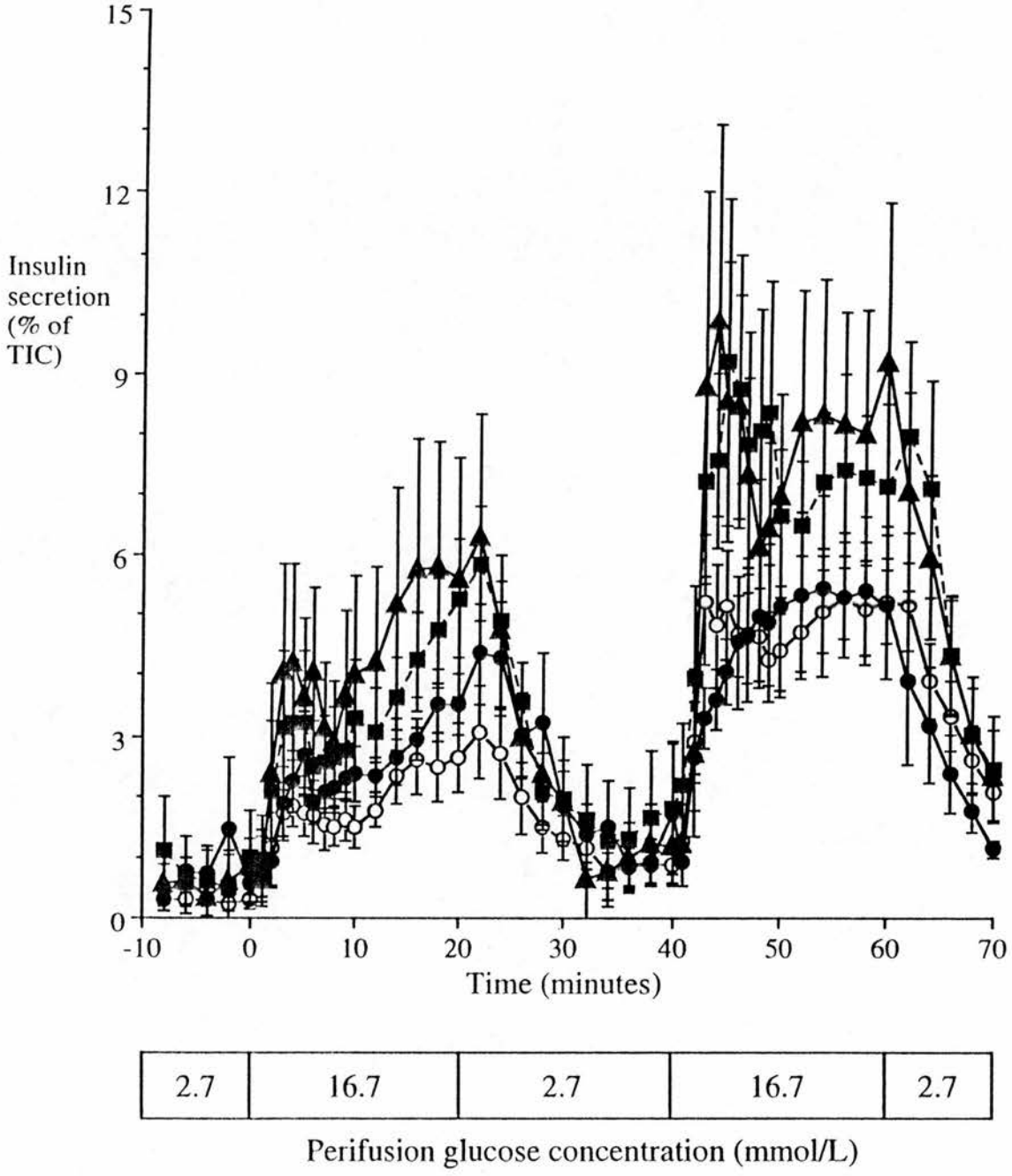
Free islets were cultured for 4 (o , n=5), 6 (■ , n=7), 9 (▲, n=7) or 13 (● , n=7) days. Numbers in brackets indicate the number of channels perifused (30 islets/channel).

expressed as peak first phase increased significantly ($p<0.05$) from culture day 4 to day 6 when considering both first and second stimulation. Peak first phase values decreased after culture day 6 and reached significance on day 9 ($p<0.01$) and day 13 ($p<0.05$) respectively. A further significant decrease in insulin secretion expressed as peak first phase following second glucose stimulation was observed from culture day 9 to day 13 ($p<0.05$). Furthermore, free islets on culture day 4 and day 6 had significantly increased peak first phase second stimulation values compared with first stimulation values ($p<0.05$ and $p<0.01$ respectively).

Figure 8 compares the effect of culture period on insulin release pattern of encapsulated rat islets during perifusion. Encapsulated islets were cultured for 2, 4, 7 and 11 days and 7 channels (30 islets/channel) were perifused at each culture period. Although a slight delay in insulin secretion was observed (maximum of 2 minutes) from microencapsulated islets upon high glucose challenge, the biphasic insulin secretory profiles of encapsulated islets were qualitatively similar to the profiles of freshly isolated and free cultured islets. Insulin secretion (% of TIC) expressed as AUC or peak first phase following first and second glucose stimulation or as total AUC was significantly lower than freshly isolated islets at all culture periods. Encapsulated islets showed optimal insulin secretion on culture day 7 whether expressed as AUC or peak first phase following first and second glucose stimulation or as total AUC. On culture day 2, insulin secretion (% of TIC) increased significantly when expressed as AUC or peak first phase following second compared with first glucose stimulation ($p<0.01$).

Table 3 compares the insulin secretion (expressed as AUC or peak first phase following first and second glucose stimulation or as total AUC) from freshly isolated free, cultured free and cultured encapsulated rat islets during perifusion. Insulin

Figure 8. The effect of culture period on insulin release pattern of encapsulated rat islets during perifusion.



Mean ± SEM

Encapsulated islets were cultured for 2 (o), 4 (■), 7 (▲) or 11 (●) days. 7 channels (30 islets/channel) were perifused at each culture period studied.

Table 3. Comparison of insulin secretion by fresh free, cultured free and cultured encapsulated rat islets during perifusion

			INSULIN SECRETION (% OF TIC)				
			Area under curve (AUC) following glucose stimulation			Peak first phase	
Islet type	Culture period (days)	n	First stimulation	Second stimulation	Total	First stimulation	Second stimulation
Freshly isolated	-	18	589.0 ± 143.3	699.8 ± 196.2	1278.8 ± 339.5	23.1 ± 0.6	44.7 ± 13.0
Free (non-encapsulated)	4	5	72.3 ± 14.1[b]	112.4 ± 18.1[b,f]	184.7 ± 32.2[b,f]	4.0 ± 0.8[b,f]	9.3 ± 1.8[a,f,k]
	6	7	125.9 ± 19.8[b]	184.0 ± 25.6[a]	309.9 ± 45.3[b]	8.1 ± 1.4[a]	16.8 ± 2.2[a,l]
	9	7	87.6 ± 16.4[b]	124.4 ± 14.4[b]	212.0 ± 30.8[b]	5.3 ± 0.8[b]	8.3 ± 1.2[a,g]
	13	7	57.0 ± 13.0[b,f]	46.6 ± 13.2[b,e,h,j]	103.6 ± 26.2[b,g,i]	4.3 ± 0.7[b,f]	4.7 ± 1.1[b,h,i]
Encapsulated	2	7	36.5 ± 8.3[c]	89.1 ± 14.3[b,d]	125.5 ± 22.6[b]	1.8 ± 0.3[b,e]	5.3 ± 1.1[b,k]
	4	7	64.1 ± 16.7[b]	136.1 ± 29.1[b]	200.9 ± 45.9[b]	3.2 ± 1.2[b,f]	9.2 ± 2.7[a]
	7	7	82.1 ± 31.2[b,f]	144.3 ± 39.0[a]	226.4 ± 70.2[b]	4.2 ± 1.6[b]	9.9 ± 3.3[a]
	11	7	46.4 ± 8.6[c]	89.1 ± 20.8[b]	135.5 ± 29.4[b]	2.7 ± 0.7[b]	5.0 ± 1.1[b]

Mean ± SEM

[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ compared with freshly isolated islets.
[d] $p<0.01$ and compared with AUC following first glucose stimulation.
[e] $p<0.05$ compared with free islets on culture day 4.
[f] $p<0.05$, [g] $p<0.01$ and [h] $p<0.001$ compared with free islets on culture day 6.
[i] $p<0.05$ and [J] $p<0.01$ compared with free islets on culture day 9.
[k] $p<0.05$ and [l] $p<0.01$ compared with peak first phase first stimulation.

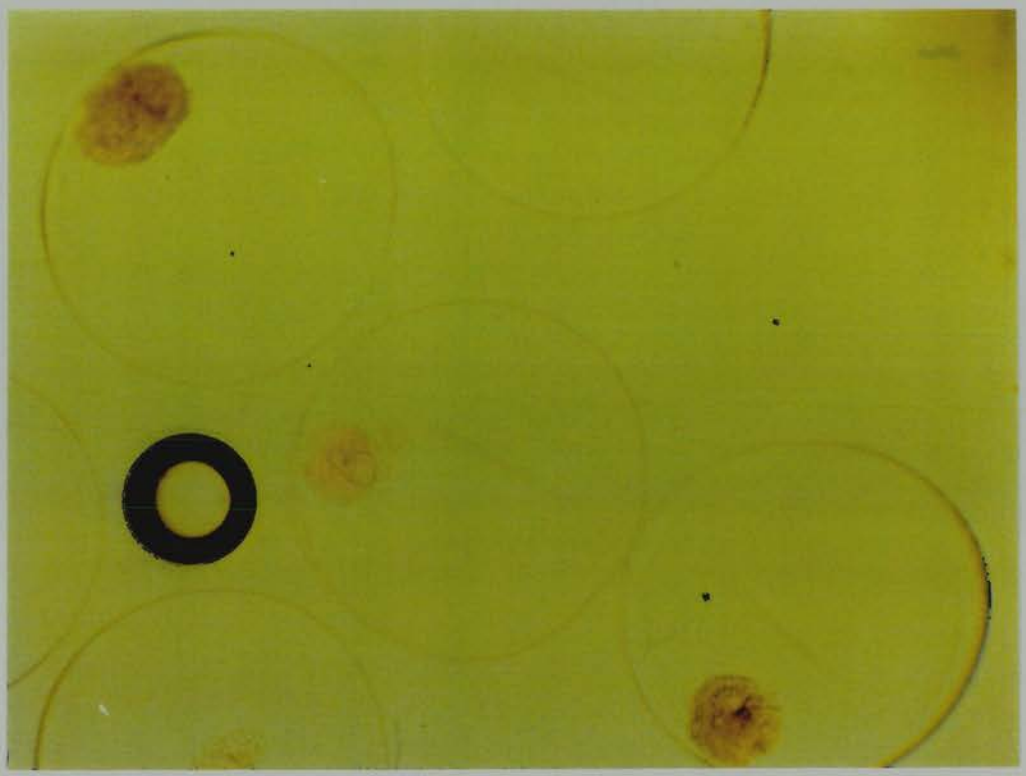
secretion from free and encapsulated cultured islets was comparable at the 4 culture periods studied although insulin secretion from free cultured islets was initially greater than insulin secretion from microencapsulated cultured islets until day 6 (optimal culture period for free islets). Indeed, free islets on culture day 4 and day 6 secreted significantly more insulin ($p<0.05$) than encapsulated islets on day 2 and day 4 respectively when considering peak first phase following first high glucose stimulation. Free islets on culture day 6 also secreted significantly more insulin than microencapsulated islets on day 4 ($p<0.05$) when considering insulin secretion as AUC following first glucose stimulation. After this culture period encapsulated islets secreted more insulin when considering AUC and peak first phase following second glucose stimulation and total AUC, although these differences were not significant.

Single rat pancreatic islets encapsulated in APA membranes are shown in Figure 9(a) following 7 days of culture at 37°C. Microcapsules were then fixed in neutral-buffered formalin and stained for insulin (b and c) and glucagon (d) as specified in Materials and Methods. Approximately 50% of islets stained positive for insulin and 33% for glucagon.

3.4. DISCUSSION

The transplantation of pancreatic islets is the most physiological way to clinically treat IDDM patients, and microencapsulation of islets has been proposed as an alternative to lifelong immunosuppression for prevention of graft rejection and recurrent autoimmune destruction. The prolonged function of allogeneic and xenogeneic islets encapsulated in APA membranes compared with free islets has been described in STZ-diabetic rats and mice (738,762). In contrast, the prolongation of islet function has been less successful in spontaneously diabetic animal models, namely NOD mice

(a)



(b)

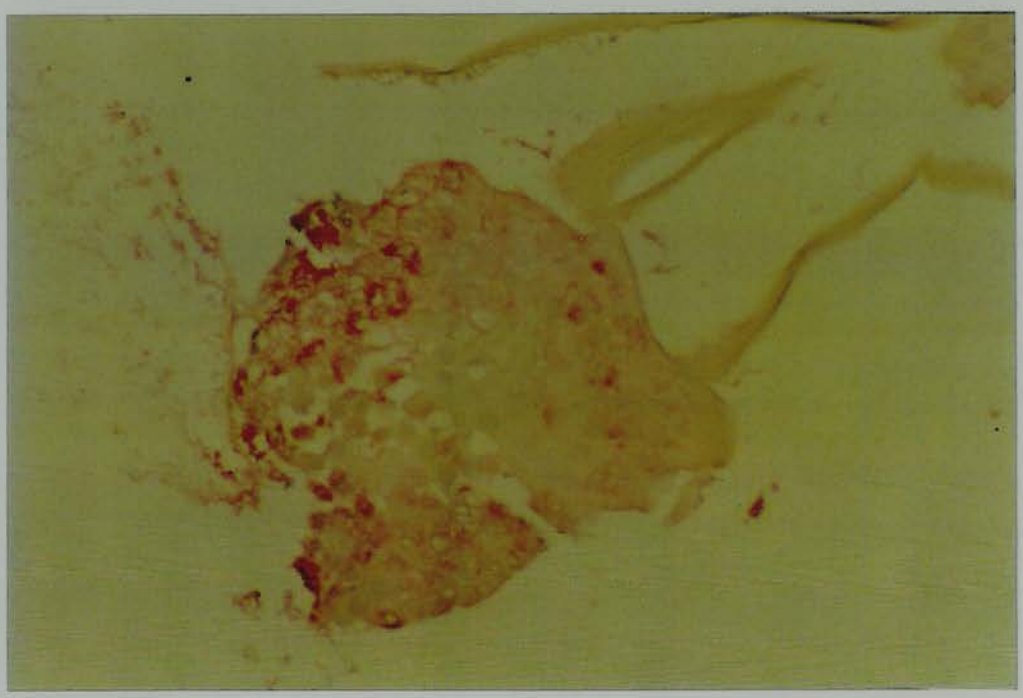
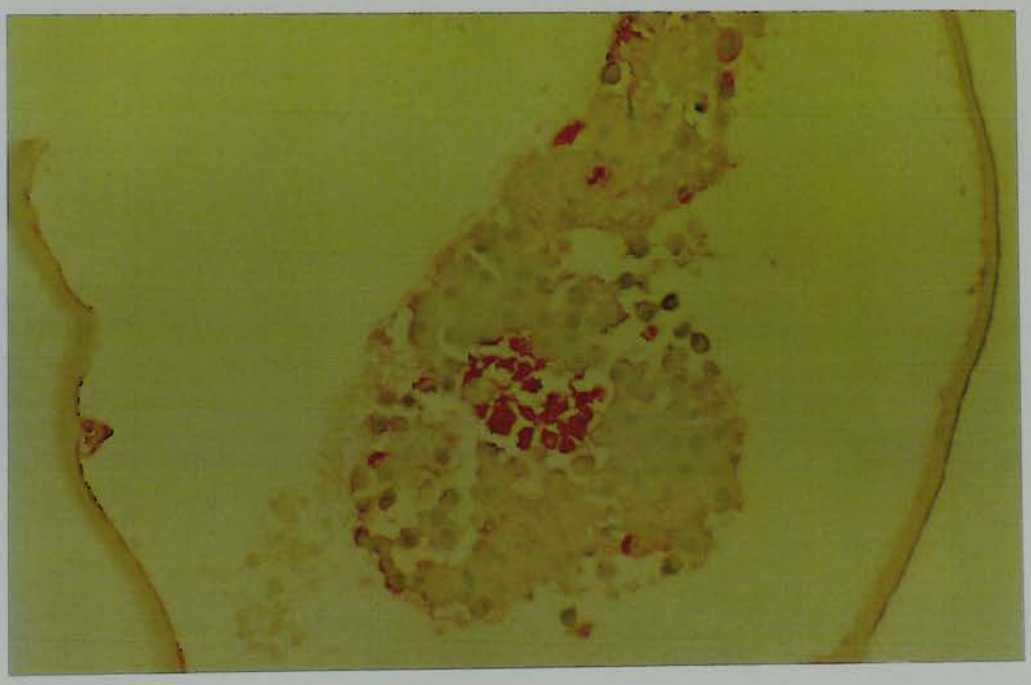


Figure 9(a). Rat pancreatic islets encapsulated in APA membrane after 7 days of culture at 37°C in a humidified atmosphere of 5% CO_2. Capsule diameter is approximately 600 µm. x 50. (b) Encapsulated rat islet after 7 days of culture stained for insulin. x 125.

(c)



(d)

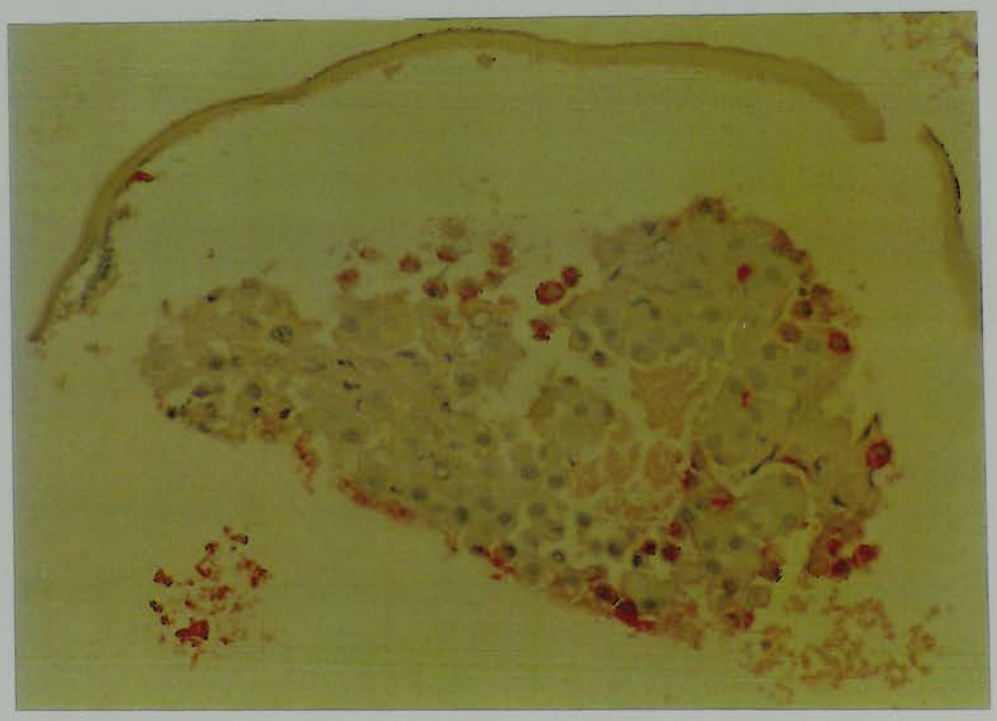


Figure 9(c). Encapsulated rat islet after 7 days of culture stained for insulin. x 125.
(d) Encapsulated rat islet after 7 days of culture stained for glucagon. x 125.

and BB rats (751,752,755,763,837). Capsule failure in all cases was ascribed to fibrotic overgrowth of the APA capsules. Only one group (750) has reported successful engraftment of microencapsulated islets in diabetic BB rats, although approximately 30% of recovered capsules exhibited cellular overgrowth and some animals required a secondary transplant to maintain relative normoglycaemia.

An essential prerequisite for successful transplantation of encapsulated islets is the maintenance of an adequate secretion of insulin in response to a glucose challenge. Results of *in vitro* testing of APA microencapsulated islets are conflicting since adequate insulin secretion has been reported by some groups (738, 736) but not others (743, 744). One explanation for these differences is the time period between encapsulation of islets and *in vitro* testing. Lim and Sun (738) cultured encapsulated islets for approximately one week prior to perifusion studies, Reach (736, 744) allowed a recovery period of approximately 18 hours, whereas Fritschy et al (743) tested the glucose-induced insulin response of microencapsulated islets immediately after completion of the encapsulation procedure.

The insulin release profiles of APA-microencapsulated rat islets, cultured for different periods of time, during perifusion studies are reported here and compared with freshly isolated and cultured free islets. All islets types gave a biphasic pattern of insulin release in response to a high stimulatory concentration of glucose (16.7 mmol/l), demonstrating that islets remained functional and viable after collagenase digestion and isolation, and microencapsulation. Glucose challenge elicited an immediate insulin response from fresh and cultured free islets whereas a slight delay (maximum of 2 minutes) was observed from microencapsulated islets. This delay in insulin release from perifused encapsulated islets in response to glucose has previously been observed by Lim and Sun (738) and Cole et al (752).

However, Lim and Sun observed a 5 minute delay in the response of microencapsulated islets to a glucose challenge. This extended delay could be due to a slower flow rate of medium through the perifusion apparatus, although the flow rate used is not stated. Horcher et al (838) observed a similar delay (2 minutes) upon challenging islets encapsulated in barium-alginate gel beads to a high glucose concentration during perifusion. In all cases, the delay was attributed to the time required for the passage of glucose across the capsule membrane to the islet and the subsequent release and passage of insulin back across the membrane and into the perifusion medium. The perifusion technique, in which insulin is continuously removed by the perifusion flow, minimises this delay and demonstrates that the APA membrane is permeable to both glucose and insulin.

The biphasic pattern of insulin release upon glucose challenge corresponds to an initial, transient and rapidly-released pool of insulin which is quickly exhausted (839) and a second phase in which insulin release is slower and prolonged (840). The first phase of insulin release may be crucial for glycaemic control as most IDDM patients do not show this initial insulin secretion (840). The secretion of insulin decreased towards basal levels after returning to a basal concentration of glucose (2.7 mmol/l) demonstrating an adequate downregulation of insulin secretion upon removal of glucose stimulation. This observation is equally important as an adequate response to increasing glucose concentrations if immunoisolated islets are to provide minute-to-minute regulation of blood glucose levels *in vivo*. Following a 20 minute " rest " period with basal glucose, islets were subjected to a second high glucose challenge. An increased first and second phase of insulin secretion was observed for all islet types. The observation that exposure of pancreatic islets to a stimulatory concentration of glucose has a priming effect on the β-cell, such that subsequent glucose challenge results in an enhanced secretion of insulin, is well established

(841-844). However, the mechanism by which priming is induced is unknown. Physiologically, islet β-cells adapt to increased demands for insulin following prolonged exposure to elevated glucose concentrations by stimulating insulin biosynthesis (845) and β-cell replication (846) thereby promoting the capacity of the β-cell to secrete insulin and correct hyperglycaemia *in vivo*. The insulin response is therefore dependent on immediate and previous exposure to glucose. Furthermore, Grill et al (844) demonstrated this priming effect after only 5 minutes exposure of isolated rat pancreas to stimulatory (27.7 mmol/l) glucose followed by a 5 minute rest period in a perifusion study. This suggests that glucose acts through mechanisms devoid of any appreciable time lag for their expression. It is therefore unlikely that priming is caused by an effect on insulin synthesis or β-cell replication, which involve lag periods of hours (847,848) or days (849) respectively before initial stimulation of these two processes increases the pancreatic content of insulin and the secretory capacity of the β-cell. The immediate stimulatory signal in the β-cell elicited by glucose exposure may alternatively involve realignment of insulin-containing secretory granules to positions more favourable for exocytosis, a reported secondary action to the effect of glucose on the microtubular-microfilamentous system of the cell (849). Translocation of insulin granules from the interior of the β-cell to its periphery upon repeated glucose stimulation will allow easier release compared with granules located further away from the site of exocytosis. This process is dependent on the presence of calcium ions (Ca^{2+}). However, controversy exists with regard to the role of Ca^{2+} in glucose priming. Grill et al (850) reported that omission of Ca^{2+} from the perifusion medium during the first exposure of islets to glucose failed to prevent the induction of priming, and subsequent responses to glucose were enhanced. In a conflicting report, Ashby and Shirling (851) stated that omission of Ca^{2+} during the first glucose challenge prevented the induction of priming in islets. More recently, Chalmers et al (842) also found that priming of islets with high glucose

concentration was only observed if Ca^{2+} were present in the perifusion medium. If Ca^{2+} were absent during the first exposure to high glucose, no enhancement of insulin release to a subsequent glucose challenge was observed. Furthermore, priming also appears to be dependent on glucose metabolism, as perifusion of islets with medium containing mannoheptulose in addition to glucose blocked priming, and D-galactose had no priming ability (850). In contrast, priming was induced by glyceraldehyde (850), suggesting that priming may involve Ca^{2+}-dependent enzymes, as well as Ca^{2+}-dependent processes such as microtubule polymerisation. Alternatively, Grill et al (844) suggested that glucose could induce priming by promoting the availability of energy in the β-cell. Insulin secretion is an energy-dependent process (852,853), therefore an energised state of the β-cell could promote insulin secretion. Indeed, ATP levels in pancreatic islets were found to be elevated following priming with high glucose in a perifusion system.

Although the insulin secretion profiles were qualitatively similar for each islet type, the actual amount of insulin secreted by the islets was quantitatively different. The glucose-induced insulin secretion from freshly isolated islets was significantly higher than that secreted from free and encapsulated cultured islets whether the data was expressed as AUC or peak first phase following first and second glucose stimulation or as total AUC. This observation has previously been reported in static culture (854,855) and perifusion systems (856). Gingerich et al (856) observed that after 24 hours of culture, glucose-stimulated insulin secretion had fallen to approximately 37% of freshly isolated islets. During the subsequent 4 days of culture, insulin secretion remained constant at 20-25% of that of freshly isolated islets. Indeed, in this study, optimal secretion of insulin from free islets (culture day 6) in response to a glucose challenge was 21-26% of that secreted by freshly isolated islets when expressed as AUC following first and second glucose stimulation or as total AUC.

The decrease in insulin release observed by Gingerich et al (856) was not due to loss of islets from the perifusion chamber since counting experiments showed that at least 90% of the islets originally placed in the perifusion system remained after 3 days of culture. Loss in insulin secretory rate with increased periods of culture may be caused by a loss of functional β-cells within islets. However, this possibility was not studied in detail by this group. An alternative explanation for the observed decrease in the insulin secreted by cultured islets was proposed by Gingerich et al (856). The presence of the hormone somatostatin, secreted by the islet δ-cells, is thought to modulate the release of islet hormones (857). The secretion of insulin from freshly isolated islets appears relatively unaffected by the inhibitory effect of exogenous somatostatin (856,858). This initial lack of inhibition by exogenous somatostatin may indicate that endogenous somatostatin may also be unable to regulate insulin secretion in the usual manner, thereby explaining the greater basal and stimulated insulin secretion observed in freshly isolated islets. The inhibitory effect of somatostatin on insulin secretion returned after culture of the same islets for 24 hours when both basal and stimulatory insulin secretory responses decreased. The later inhibitory mechanism of somatostatin is unknown and a recovery period to allow repair and regeneration of cellular components that were adversely affected during the isolation procedure appears necessary.

Insulin secretion from free and encapsulated cultured islets in response to a high glucose challenge was similar when compared at the four culture periods studied. Lum et al (766), Cole et al (752) and Lim and Sun (738) also reported comparable insulin secretion from free and encapsulated islets in response to high and low glucose concentrations in perifusion studies after overnight, 24 - 48 hours and approximately 1 week culture respectively. Lim and Moss (859) also showed that the amounts of insulin released from free and microencapsulated islets by a

combination of high glucose and theophylline were comparable in perifusion studies. However, after 24 days of culture, perifusion of free islets showed a high level of baseline insulin release and an abnormal stimulation-response pattern. In contrast, microencapsulated islets produced a reasonably low level of baseline insulin release and a typical biphasic stimulation-response pattern. Abundant and continued cell proliferation was evident in all encapsulated islets and viable β-cells were observed upon staining with haematoxylin and eosin and with aldehyde-fuchsin. Microencapsulated islets continued to respond to glucose stimulation during an 8 week period. In contrast, free islets showed signs of degeneration, including progressive β-cell degranulation at the end of 3 weeks.

These observations have been confirmed in long-term static incubation studies (860) although the normal biphasic pattern of glucose-induced insulin secretion cannot be evaluated using this technique. Free cultured rat islets initially secreted significantly more insulin into the medium than encapsulated cultured islets on day 1. On day 3 this difference was no longer significant. By day 10 a significantly higher insulin concentration was detected in the medium bathing the encapsulated islets. In this study, secretion of insulin from microencapsulated islets upon high glucose challenge was optimal after a recovery culture period of 7 days whereas insulin secretion from free cultured islets was optimal on culture day 6 and decreased thereafter. This decrease in insulin secretion from free cultured islets may be due to the disintegration, i.e. detachment, of β-cells as observed by Leung et al (860) after 2-3 weeks of culture at 37°C. Throughout the remaining 8 week incubation period, encapsulated islets consistently secreted more insulin than free islets. Indeed, Lim and Sun (738) observed a normal degree of β-cell granulation in microencapsulated islets cultured for as long as 13 weeks. Cole et al (752) observed that static insulin release from encapsulated rat islets decreased to 35% following 6 weeks culture. Although a

greater reduction was observed from free islets after approximately 18 days, significance was not achieved until week 6.

In contrast, Chicheportiche and Reach (744) and Fritschy et al (743) reported poor, sometimes absent, insulin response to a glucose challenge using encapsulated islets. Chicheportiche and Reach concluded that the insulin secretory response correlated with the volume of the microcapsule since small (350 μm) capsules responded rapidly to glucose stimulation in a static incubation whereas no response was observed with larger capsules (650 μm). Diffusion across the microcapsule membrane is known to decrease as the capsule diameter increases and is dependent on the difference in insulin concentrations across the membrane in static incubations. Since islets contained in large and small capsules secrete the same amount of insulin in response to glucose stimulation, intracapsular insulin concentration will be considerably lower in large capsules due to their larger volume. This may explain the slower insulin release from larger capsules. *In vivo*, Lum et al (766) observed prolonged reversal of the diabetic state in NOD mice after transplantation of rat islets encapsulated in smaller (250-350 μm) APA microcapsules. Advantages of these smaller capsules *in vivo* include increased islet viability since oxygen and nutrients are more accessible to the cell across the reduced dead space of the capsule. This also enables faster β-cell response to glucose fluctuations in the recipient, an important consideration for clinical application of microencapsulated islets in IDDM patients. Kinetic modelling of glucose homeostasis in man indicates that the lag time of increased insulin delivery in response to a glucose load by an artificial pancreas must be shorter than 15 minutes to avoid overexcursion of postprandial blood glucose levels (861). Further advantages of using smaller capsules in transplantation studies include a reduced graft volume and greater mobility of capsules, which may lessen the chance of mechanical damage after implantation. Reduced tissue irritation may

consequently decrease cell overgrowth on capsule surfaces thus prolonging the survival of microencapsulated islet grafts *in vivo*.

However, Fritschy et al (743) reported that the presence of the microcapsule could not explain the decreased insulin secretion by encapsulated islets since the response was similarly decreased when islets were encapsulated without the final membrane-forming step. Instead, this group observed a severely reduced insulin release after prolonged suspension of islets in saline or treatment with citrate during the encapsulation procedure, possibly as a result of damage to the islet secretory system. Replacement with Ca^{2+}-free Krebs-Ringer bicarbonate buffer and 1 mmol/l EGTA respectively improved insulin release significantly. Lévesque et al (862) also reported that decreased glucose-induced insulin secretion from freshly microencapsulated islets compared with free islets could not be explained by the presence of the microcapsule since this decrease was also observed using microencapsulated islets that had been mechanically freed from their capsules prior to *in vitro* testing. This group investigated static insulin and glucagon secretion from microencapsulated islets in response to long-term exposure to glucose. Glucagon secretion was examined as glucagon-secreting α-cells are located in the periphery of islets and are thus more susceptible to damage during encapsulation than other islet cells. Glucose-induced insulin secretion was significantly reduced from islets 48 hours after microencapsulation compared to free cultured islets. In contrast, secretion of glucagon was similar in the two groups of islets. Differences in insulin diffusion properties of the capsule membrane were eliminated since islets mechanically freed from their capsules also exhibited reduced insulin, but normal glucagon, secretion in response to high glucose stimulation. These findings further demonstrate the permeability of the APA microcapsule to small molecules such as insulin, glucagon and glucose. Analysis of islets immediately after encapsulation demonstrated

significant reductions in both insulin and glucagon content, possibly due to damage during the encapsulation procedure or by significant stimulation of secretion of both hormones by one of the reactant solutions used to create the microcapsule. Further reductions in the insulin and glucagon contents of both free and encapsulated islets were observed over a 5 day culture period, although the loss of both hormones from microencapsulated islets was less than that from free cultured islets. The insulin content of microencapsulated islets remained significantly lower than free islets thereby accounting for the lower secretion of insulin observed in encapsulated islets. However, islet glucagon content became identical in free and microencapsulated islets during the 5 day culture period. Thus, although microencapsulation induced an immediate loss of islet insulin and glucagon content, the microcapsules seemed beneficial over long-term culture by diminishing further hormone loss, as observed with free cultured islets, by preventing islet disintegration.

In this study, insulin secretion from free and encapsulated islets upon glucose challenge were comparable after defined periods of culture. Thus, although islets may initially show a poor, sometimes absent, glucose-stimulated insulin response following microencapsulation (either as a result of islet damage by certain reactants used to create the capsule or by significant reductions in the insulin content of islets following microencapsulation), a period of culture (optimal after 7 days) allowed recovery of insulin-secreting function. This suggests that the deleterious effect of the encapsulation procedure, as originally described by Lim and Sun (738), is at least partially reversible. The recovery period may allow time for repair and regeneration of cellular components of the islet affected by the encapsulation procedure. This observation is particularly important for clinical transplantation of microencapsulated pancreatic islets.

Microencapsulation of pancreatic islets in APA membranes also provides a favourable microenvironment for long-term *in vitro* maintenance of β-cell function compared with free cultured islets. A normal degree of β-cell granulation in microencapsulated islets is observed during long-term culture whereas free islets show signs of degeneration and progressive β-cell degranulation. This suggests microencapsulated islets may be clinically advantageous compared with free islets for islet transplantation in the diabetic patient. However, several problems remain to be solved before microencapsulated islet transplantation becomes a realistic option for treatment of IDDM. These problems include modification of the composition and purification of alginate to improve capsule strength and biocompatibility respectively, and determination of microcapsule diameter, graft volume and transplantation site for optimal function of microencapsulated islets. The recent report by Soon-Shiong et al (761) describing insulin-independence in an immunosuppressed IDDM patient after encapsulated human islet transplantation is encouraging although further trials to determine the optimal dose of encapsulated islets necessary to achieve insulin-independence in non-immunosuppressed patients are required.

CHAPTER 4

PREVENTION OF RECURRENT DIABETES IN SPONTANEOUSLY DIABETIC, INSULIN-DEPENDENT AUTOIMMUNE BB/E RATS AFTER ISLET TRANSPLANTATION BY ANTI-CD4 AND ANTI-CD8 MONOCLONAL ANTIBODY THERAPY.

4.1. INTRODUCTION

The potential of isolated pancreatic islet transplantation as a treatment and possible cure of IDDM is evident. In contrast to exogenous insulin therapy, islet transplantation restores physiological control of glucose homeostasis and may also prevent the secondary long-term complications and morbidity associated with IDDM. However, two immune responses jeopardise the success of islet transplantation in subjects with IDDM : allograft rejection and recurrence of the β-cell-specific autoimmune process responsible for primary disease onset. T lymphocytes are the principal effectors of all immune responses and must therefore be " controlled " to ensure successful transplantation of an islet allograft.

Conventional immunosuppression, including CsA, comprimises the entire immune system, involves long-term administration, exhibits limited efficacy and risks infection and drug toxicity. The alternative approach involving immunoisolation of islets has failed due to the bioincompatibility of materials used to form the protective membranes. An improved and more sophisticated approach to immunosuppression is clearly necessary. Ideally, immunosuppression would control the T lymphocytes specifically responsible for initiation of the immune response without affecting the remainder of the immune system. A major advantage of moAb therapy is specificity for target antigens. Indeed, since collaboration between T cells and several other cell types is essential for function of the immune system, interruption of these collaborative events should result in therapeutic immunosuppression. The ability to induce specific immunological unresponsiveness in the adult would have major implications for both tissue grafting and treatment of autoimmune diseases. $CD4^+$ and $CD8^+$ T lymphocytes have been targeted by depleting and non-depleting moAb because of the central role of these cells in the induction and maintenance of immune

responses. However, studies in the rat utilising anti-CD4 and anti-CD8 immunotherapy to prevent allograft rejection have reported variable results. In this study, the effect of short-term administration of depleting or non-depleting moAb specific for the helper ($CD4^+$) or cytotoxic/suppressor ($CD8^+$) T lymphocyte subsets on survival of islets transplanted intraportally in the spontaneously diabetic, insulin-dependent autoimmune BB/E rat was investigated.

4.2. MATERIALS AND METHODS

4.2.1. ANIMALS

4.2.1.1. The BB/E rat

The BB/E rat has been previously described in this thesis. The mean age at onset of diabetes was 108 $\pm$ 3 days and the mean duration of diabetes at the start of treatment was 117 $\pm$ 3 days in this study.

4.2.1.2. The Wistar and Wistar Han albino rat

At the time of this study, breeding and use of normal Wistar rats had been replaced by outbred Wistar Han (WH) rats in the Biomedical Research Facility. RT1-serotyping of WH rats indicated that the gene frequencies for the *d*, *u*, *l* and *c* haplotypes were 50%, 30%, 15% and 5% respectively. The supply of WH rats was supplemented by normal Wistar rats supplied by Harlan Olac Ltd., Bicester, Oxon, UK. No RT1-serotyping was available on these outbred rats.

4.2.1.3. The Balb/c mouse

Female Balb/c mice were bred and maintained in the Biomedical Research Facility and used for the raising of ascites at 6-8 weeks of age.

4.2.2. HYBRIDOMA CELL LINES

The three hybridoma cell lines used (Table 4) were obtained from the European Collection of Animal Cell Cultures (ECACC), Division of PHLS Centre for Applied Microbiology and Research (Salisbury, Wiltshire, UK) and were grown in culture or as ascites.

4.2.2.1. Culture of hybridoma cell lines

Hybridomas were received frozen in solid CO_2 and were thawed in 0.5 ml supplemented RPMI-1640 prewarmed to 37°C. Each cell line was transferred to a sterile 50 ml Corning centrifuge tube, washed and harvested by centrifugation (75 x g, 5 minutes) at 20°C. Cells were resuspended in supplemented RPMI 1640 and cultured in 25 cm^3 Falcon tissue culture flasks (Becton Dickinson) in a humidified atmosphere of 5% CO_2 at 37°C. All cells were maintained at a concentration of approximately 5 x 10^5 cells/ml. The growth of the W3/25 moAb-secreting hybridoma cell line in supplemented RPMI 1640 medium was poor and was significantly improved in Iscoves Modified Dulbecco's medium containing 25 mmol/l glucose (Sigma) supplemented with 0.3% sodium bicarbonate, 0.1% BSA, 25 mmol/l Hepes, 4 mmol/l L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin.

Table 4 : Characteristics of moAb used

MoAb	ECACC clone	Isotype	Specificity	Reference
W3/25 (non-depleting)	84112002	IgG1	CD4 : thymocytes, helper T cells and macrophages	863
OX8 (depleting)	84112009	IgG1	CD8 : thymocytes, cytotoxic/suppressor T cells and NK cells	864
OX38 (depleting)	88051303	IgG2a	CD4 : thymocytes, helper T cells and macrophages	665

4.2.2.2. Determination of viability and concentration of cells

Viability and concentration of cells were checked daily using 1 volume of cell suspension mixed with 1 volume of a 10 µl/ml solution of acridine orange (Sigma) and ethidium bromide (Sigma). The cells were placed in a haemocytometer (Hawksley Cristalite, Hawksley, UK) and viewed through a mercury lamp fluorescent microscope (Leitz, Wetzlar, Germany) using UV and visible illumination simultaneously. Viable cells took up acridine orange and appeared green, whereas dead cells took up ethidium bromide and appeared orange.

4.2.2.3. Collection of monoclonal antibodies

Antibodies were collected daily by centrifugation (2500 x g, 10 minutes) of cells at 20°C. The pelleted hybridoma cells were resuspended to the appropriate concentration in fresh supplemented RPMI 1640 and returned to culture, whilst the immunoglobulin present in the supernatant was precipitated using saturated (0.9 g/ml) ammonium sulphate (SAS), pH 7.4 (Sigma). SAS was added slowly and with stirring to the supernatant until 40% saturation was achieved. Following overnight incubation at 4°C, precipitate was recovered by centrifugation (2500 x g, 30 minutes), washed twice and resuspended in two volumes of 40% SAS and stored at 4°C.

4.2.2.4. Raising of ascites

Ascites were raised in female Balb/c mice in addition to growing of hybridomas in culture. Mice were primed with 0.8 ml of pristane (2, 6, 10, 14-tetramethyl-pentadecane) (Sigma) 7 days prior to intraperitoneal injection of 2 - 5 x 10^6

hybridoma cells in 0.5 ml of sterile phosphate-buffered saline (PBS; 140 mmol/l sodium chloride; 8 mmol/l di-sodium hydrogen orthophosphate; 2.7 mmol/l potassium chloride and 1.5 mmol/l potassium dihydrogen orthophosphate; BDH). Ascites were drained 14 days after immunisation and again after killing of the animal by inserting a 19G needle into the peritoneal cavity and draining the fluid into a sterile universal container under gravity. The solution was centrifuged (300 x g, 20 minutes) at 20°C and the supernatant washed three times to remove the pristane layer, prior to the precipitation of immunoglobulins in 40% SAS.

4.2.2.5. Determination of protein (antibody) concentration

The protein (antibody) concentration of each precipitate after centrifugation and resolubilisation in sterile PBS was determined regularly by spectrophotometry (Pye Unicam PU8610 UV/VIS kinetics spectrophotometer, Pye Unicam Ltd., Cambridge, UK) based on an absorbance of 0.69 for a 1 mg/ml protein solution at 280 nm using PBS as a zero blank. After a sufficient quantity of each antibody had been generated, the precipitates were purified and concentrated by ultrafiltration.

4.2.2.6 Ultrafiltration of monoclonal antibodies

Precipitates were centrifuged (2500 x g, 10 minutes) at 20°C and the pellets resolubilised in 50 ml of PBS. Undissolved material was removed by centrifugation and the supernatants sterile-filtered through Falcon 0.45 μm cellulose acetate membrane filters (Becton Dickinson) prior to transfer to a sterile ultrafiltration stirred cells (model 8050, Amicon Ltd., Gloucestershire, UK). A sterile Diaflo ultrafiltration filter (membrane type YM 30; Amicon) with a 30 000 molecular weight cut-off was appropriate for elution of toxic SAS under pressure (30 psi) and

retention of antibody in the stirred cell. Each antibody was washed with 9 volumes of sterile PBS and slowly concentrated to a final volume of 10 ml. The concentrated solutions were transferred to sterile 20 ml universal containers (Sterilin, Bibby Sterilin Ltd., Staffordshire, UK) and the final protein concentrations determined using PBS as a zero blank. All antibodies were diluted in sterile PBS to a final concentration of 10 mg/ml, and 1 ml aliquots were transferred to sterile 5ml glass vials (Monoject, Sherwood Medical Industries Ltd., West Sussex, UK). Samples were then lyophilised (ChemLab Freeze-drier, ChemLab Instruments Ltd., London, UK) to prevent bacterial and fungal growth during storage at -20^{o}C.

4.2.3. MONOCLONAL ANTIBODY TREATMENT

4.2.3.1. Effect of moAb dose *in vivo*

Established diabetic BB/E rats maintained on CIT were randomly divided into 5 treatment groups : (1) PBS-treated, i.e. controls (n = 3); (2) W3/25 moAb-treated (n = 3); (3) OX8 moAb-treated (n = 3); (4) OX38 moAb-treated (n = 3) and (5) treated with a combination of OX8 and OX38 moAb (n = 3). Rats were bled (approximately 1 ml) by tail-tipping prior to subcutaneous injection of a priming dose (10 mg) of the appropriate moAb on day -3. Blood samples were collected 60, 120 and 240 minutes and 24 hours (i.e. day -2) after injection. Animals were subsequently injected with daily maintenance doses (1 mg) of moAb from days -2 to day 10. Animals were bled again during moAb treatment on day 0 (the day of islet transplantation for experimental diabetic BB/E rats), the last day of treatment (i.e. day 10) 1 week post-moAb treatment and 2 weeks post moAb-treatment at which time animals were killed. Lymphoid tissues were also collected and processed

for immunofluorescence analysis. In addition, blood samples were used in Con A proliferation assays.

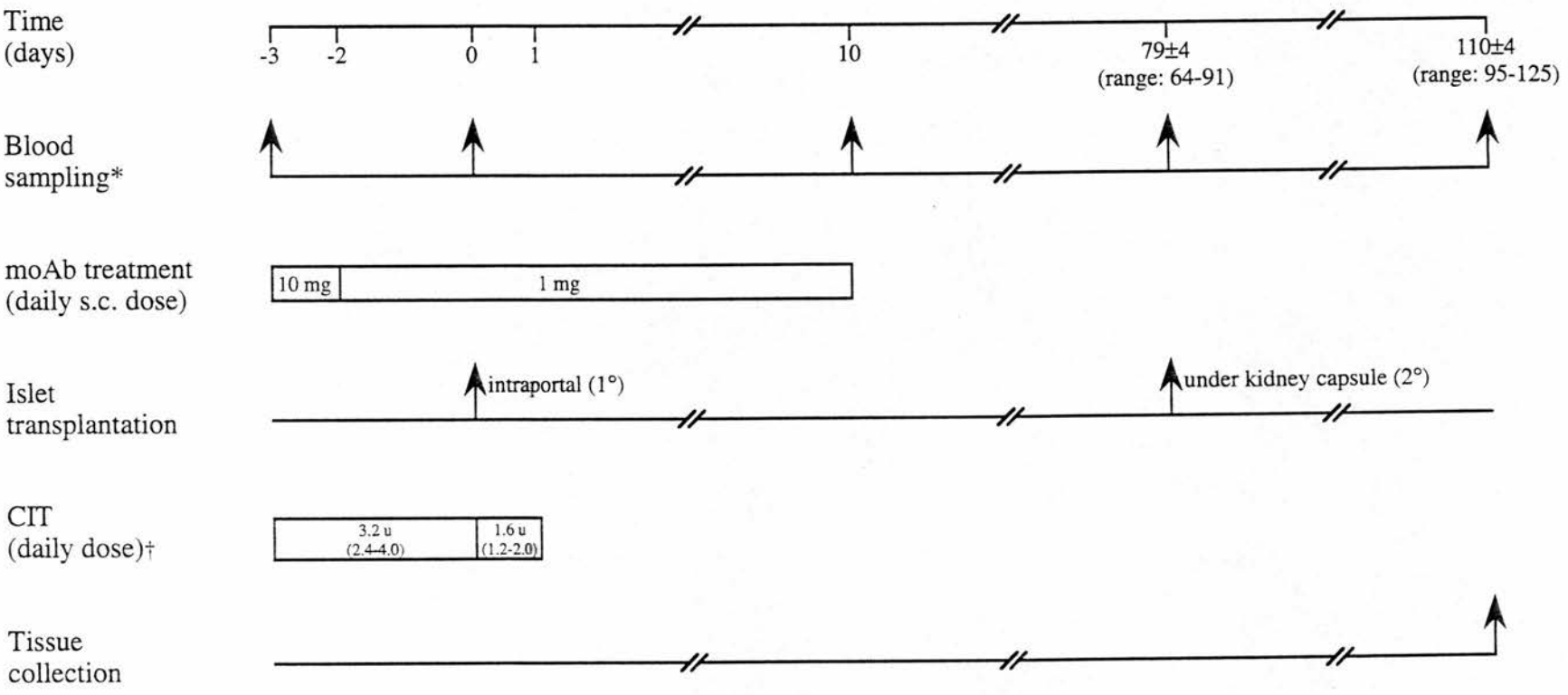
4.2.3.2. Experimental protocol

Established diabetic BB/E rats maintained on CIT were randomly divided into 5 treatment groups : (1) PBS-treated, i.e. controls (n = 8); (2) W3/25 moAb-treated (n = 9); (3) OX8 moAb-treated (n = 9); (4) OX38 moAb-treated (n = 9) and (5) treated with a combination of OX8 and OX38 moAb (n = 9). Either 1 mg or 10 mg of each moAb was dissolved in 0.5 ml sterile PBS and injected subcutaneously in the nape of the neck at the same time every day (0930-1100). On day -3, animals were injected with 10 mg of the appropriate moAb and subsequently with 1 mg for the remaining 13 days of treatment. Diabetic BB/E rats were maintained on CIT from day -3 until day 0 when half the usual dose of insulin was administered prior to receiving an intraportal islet graft. After the islet graft the animals received no further insulin treatment. Peripheral blood samples were taken pre-moAb treatment (day -3), prior to transplantation of the primary (1°) islet graft (day 0), at the end of moAb treatment (day 10), upon failure of the 1° islet graft and at death, at which time lymphoid tissues were also collected and processed for immunofluorescence analysis. Animals maintaining a long-term functioning 1° islet graft were also bled prior to transplantation of a secondary (2°) islet graft under the kidney capsule. The experimental protocol is summarised in Table 5.

4.2.4. ISOLATION OF PANCREATIC ISLETS

At the time of this study, breeding and use of normal Wistar rats had been replaced by outbred WH rats in the Biomedical Research Facility. WH rats are anatomically

Table 5. Experimental protocol for islet-transplanted diabetic BB/E rats receiving moAb treatment.



* Blood samples were also collected from diabetic BB/E rats upon detection of islet graft failure and tissue samples subsequently collected.

† mean (range)

immature when compared to weight-matched Wistar rats, thus insulin secretion by islets isolated from both rat types in response to glucose was evaluated by a static incubation technique. Wistar rats weighing approximately 250 g (n = 2) and WH rats weighing approximately 280 g (n = 2); 315 g (n = 2); and 375 g (n = 2) were used for the isolation of islets. The method for the isolation of pancreatic islets was as previously described except that digests were separated from contaminating exocrine elements using a four-layer discontinuous BSA gradient (Advanced Protein Products, West Midlands, UK) at the same densities as previously used for the dextran gradients. BSA is reported to be less toxic to islets than dextran (865).

4.2.4.1. Static incubation

For each group of animals, isolated islets were transferred to a sterile 4 well multidish (Nunclon Delta, Denmark) (20 islets/well), suspended in 1 ml of glucose-free RPMI 1640 supplemented media and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2. The supernatants were removed (approximately 900 µl) and replaced with an equivalent volume of fresh media containing 11.1 mmol/l glucose, and islets cultured for a further 2 days. Supernatants were subsequently removed and stored at -20°C until assay of insulin. Islets were washed three times with glucose-free RPMI 1640. During each wash, islets were left in the medium and maintained at room temperature for 10 minutes to remove insulin which may have accumulated during the previous culture period. After completion of washing, prewarmed (37°C) RPMI 1640 medium containing 2.5 mmol/l glucose (non-stimulatory) was added to each well and the plate returned to the incubator for 90 minutes. Supernatants were then removed and replaced with RPMI 1640 medium containing 20 mmol/l glucose (stimulatory) and the plate incubated for 90 minutes. Islets were washed three times after removal of supernatant and re-incubated in

non-stimulatory medium for 90 minutes. Supernatants were then removed and islets were washed three times with 0.9% saline and suspended in 500 µl distilled water prior to sonication. TIC was assessed as previously described.

4.2.5. ISLET TRANSPLANTATION

4.2.5.1. Intraportal route

A 3 cm long midline abdominal incision was made under fluothane anaesthesia. Displacement of the large and small bowel to the left side of the animal revealed the portal vein. Blood supply was restricted by a steel wire placed underneath the portal vein and allowed to sit across the wound. 1200-1600 pooled islets, cultured for 5-6 days and washed four times in sterile PBS prior to transplantation, were drawn into a 1 ml syringe (via a 23G needle) in a minimal volume of PBS and injected slowly into the portal vein. Care was taken to avoid the introduction of air into the portal system. Blood was withdrawn into the syringe and re-injected to ensure transfer of any residual islets. The needle was removed and pressure applied over the venipuncture for approximately 3 minutes using a cotton bud by which time bleeding had usually ceased. The steel wire was removed from beneath the portal vein, allowing blood flow to carry the islets forward into the liver. After returning the bowel to its original position, the wound was closed with 3/0 braided silk suture (Mersilk, Ethicon Ltd., Edinburgh, UK) and the outer skin with Michel suture clips (Northern Hospital Supplies, Edinburgh, UK).

After islet transplantation, rats from all groups were observed in metabolic cages and allowed free access to food and water. Blood glucose concentration was initially determined daily to assess the immediate function of the islet graft and subsequently

measured twice weekly upon stabilisation of glycaemic control. Blood glucose level was assessed between 0930 and 1100 and values during this period were similar to those determined at peak feeding time (2200) as determined in Chapter 2 of this thesis. In addition, body weight was measured and urine samples checked for the presence of glucose and ketones twice weekly.

Animals maintaining normoglycaemia for 64-91 days (mean ± SEM : 79 ± 4 days) post-transplant received a second graft of approximately 500 islets implanted under the kidney capsule to determine whether induction of tolerance to islet tissue had been achieved. The islets for both the intraportal and kidney capsule transplants were isolated from the same donor species and cultured 5-6 days prior to transplantation.

4.2.5.2. Kidney capsule site

Under halothane anaesthesia, rats were shaved over the left loin and a small incision made after swabbing the area with alcohol. The left kidney was exposed by gentle traction and a small incision through the kidney capsule (approximately 2 mm long) was made at the upper pole of the lateral border of the kidney using a scalpel blade. The capsule was then raised from the kidney surface by inserting a round-ended mouth gavage needle. Islets suspended in a minimal volume of PBS were drawn up into a Portex cannula attached to a 1 ml syringe and transferred slowly into the space created under the kidney capsule. The kidney was kept moist with saline throughout, and upon completion of islet transfer, the wound was closed with 3/0 braided silk suture and the outer skin with Michel suture clips. Animals were killed 31 ± 3 days later. Untreated insulin-dependent diabetic BB/E rats (n = 6) transplanted with approximately 500 islets under the kidney capsule alone acted as controls. CIT was administered as required in order to maintain a degree of normoglycaemia.

Islet grafts were considered to have failed after 3 consecutive blood glucose concentrations of >13.1 mmol/l in conjunction with consistent weight loss and/or the presence of glycosuria. After absolute graft failure, animals were killed by CO_2 asphyxiation, and all lymphoid tissues were removed from animals using standard surgical techniques. After preparation and determination of cell concentrations, cells were used for immunofluorescence analysis. Peripheral blood lymphocytes (PBL) were also used in an *in vitro* mitogen stimulation assay.

4.2.6. PREPARATION OF LYMPHOID TISSUE

4.2.6.1. Peripheral blood lymphocytes

Heparinised blood samples collected from the tails of non-anaethetised rats were transferred to a sterile 1.5 ml Starstedt tube and centrifuged (3500 x g, 3 minutes). Larger volumes of blood (5 ml) were obtained by cardiac puncture prior to killing and collected in a sterile 20 ml tube containing heparin. Peripheral blood lymphocytes were isolated by centrifugation (560 x g, 10 minutes) at 20°C. The buffy coat was transferred to a sterile 15 ml Corning centrifuge tube and washed with 9 volumes of sterile PBS supplemented with 0.1% BSA. Contaminating red blood cells were lysed by addition of 9 volumes of freshly prepared sterile Tris-ammonium chloride solution, pH 7.2 (9 volumes of 160 mmol/l ammonium chloride mixed with 1 volume of 170 mmol/l Tris [hydroxymethyl] methylamine) to the pelleted cells for 10 minutes at room temperature. The procedure was repeated after centrifugation of cells (560 x g, 10 minutes) at 20°C. Cells were washed three times and harvested between each wash by centrifugation (560 x g, 10 minutes) at 20°C. Cells were washed and harvested by centrifugation (560 x g, 10 minutes) at 20°C unless otherwise stated.

4.2.6.2. Splenocytes

The spleen was processed in FACS-PBS medium (PBS supplemented with 0.1% BSA Fraction V, 15 mmol/l sodium azide [Sigma] and 0.5 mmol/l EDTA [BDH]) by teasing the spleen apart using forceps and a scalpel blade. The disrupted tissue was homogenised by hand and the resultant cell suspension was lysed of red blood cells by the same procedure described in the preparation of PBL, with the exception that FACS-PBS was used for the washing steps.

4.2.6.3. Peripheral and mesenteric lymphocytes and thymocytes

Peripheral and mesenteric lymph nodes (PLN and MLN respectively) and thymic tissue were processed in FACS-PBS by mincing the tissue finely with scissors prior to gentle homogenisation. The resultant cell suspension was washed once in FACS-PBS.

4.2.6.4. Peritoneal exudate cells

10 ml of FACS-PBS were injected intraperitoneally prior to opening up the peritoneal cavity and retrieving the maximum volume of medium containing peritoneal exudate cells (PEC) using a plastic pipette. The cells were washed once in FACS-PBS.

4.2.7. IMMUNOLABELLING OF CELL SURFACE ANTIGENS

Cell surface phenotype was assessed using aliquots of 1 x 10^6 cells from processed tissues. Cells were incubated with 20 µl of conjugated moAb (see Table 6) for 40 minutes at 4°C. All conjugated moAb were pretitrated using 1 x 10^6 lymphocytes

Table 6 : Conjugated moAb used for immunolabelling.

MoAb	Clone	Specificity	Source	Reference	*Volume µl/well
Mouse anti-rat CD8a (PE)	OX8	see Table 4	Pharmingen, San Diego, CA,USA	866	0.075
Mouse anti-rat CD4 (FITC)	OX38	see Table 4	Pharmingen,	665	0.01
Mouse anti-rat CD5 (PE)	OX19	Thymocytes, peripheral T cells	Pharmingen,	867	0.20
Rabbit anti-rat IgM (FITC)	-	B lymphocytes	Serotec, Oxford, UK	-	0.15

Key

PE : phycoerythrin
FITC : fluorescein isothiocyanate

*volumes indicated were suspended in 20 µl FACS-PBS medium for staining 0.25 - 1 x 10^6 cells.

isolated from peripheral and mesenteric lymph nodes of WH donors to establish optimal conditions for FACS analysis. Unbound antibody was removed by washing twice with FACS-PBS and fixed in 300 µl of FACS-PBS and 10% formalin (2 : 1) prior to immunofluorescence analysis.

4.2.8. IMMUNOFLUORESCENCE ANALYSIS

Immunofluorescence analysis was performed by Andrew Sanderson at the Department of Zoology, University of Edinburgh using a Becton Dickinson FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, California, USA) fitted with an argon-ion laser (excitation wavelength 488 nm). During FACS analysis, cells in suspension pass from a reservoir through a nozzle assembly, become centred in a cell-free sheath fluid, and pass through a focused laser beam (868). Light is scattered by the cells and molecules are excited and fluoresce. Fluorescent light is collected and focused, and a dichroic reflector directs defined wavelength ranges to separate photomultiplier detectors which convert light signals to electronic signals.

Where overlap in emission spectra of fluorescent dyes occurred, as for phycoerythrin (PE) and fluorescein isothiocyanate (FITC) used in this study, it was necessary to carry out a process of 2-colour compensation to obtain independent dye measurements. This was accomplished by comparison with standard solutions of each cells labelled with either PE- or FITC-conjugated antibody alone, and adjusting the output of samples to zero for the inappropriate dye.

Light scattered by cells is generally collected in the forward-scatter (FSC) range of 3^{o}-12^{o}, and in the side-scatter (SSC) range of 70^{o}-110^{o}. Light scatter is most proportionate to cell size at low scatter angles (i.e. FSC) whilst at large scatter

angles (i.e. SSC) the signals tend to reflect the fine internal structure and granularity of cells (868). During data collection (10 000 cells), red cells and dead cells were routinely gated out according to their low FSC values which tend to be significantly lower than other cells. During data analysis, non-lymphocyte cells (including macrophages and granulocytes) were gated out according to their large SSC which is substantially higher than for the lymphocyte population. Data was analysed using dot plots to determine % lymphocytes and lymphocyte subsets.

4.2.9. MITOGEN STIMULATION ASSAY

Initially, MLR was considered as a standardised method to assess the responsiveness of PBL from moAb-treated BB/E rats to foreign islet tissue from donor Wistar or WH rats. This method was unsuitable due to the highly variable response of BB/E PBL even before treatment. For this reason, a mitogen (Con A) stimulation assay was chosen to monitor the effectiveness of moAb treatment with time. PBL were harvested and resuspended in supplemented RPMI 1640, and aliquots (2.5 x 10^5 cells) were cultured in 96-well round-bottom microculture plates (Corning Cell Wells) with or without Con A at a final concentration of 2.5 µg/ml. The final sample volume was 200 µl and samples were analysed in triplicate. The proliferation assays were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 88-96 hours and pulsed with 1 µCi ^{3}H-thymidine (specific activity : 5 Ci/mmol, Amersham) per well during the final 24 hour period. Cells were collected onto filter mats (Skatron Instruments Ltd., Suffolk, UK) using a semi-automated Combi cell harvester (Skatron) and ^{3}H-thymidine incorporation was assessed by liquid scintillation counting using a 2000CA Tri-Carb liquid scintillation analyser (Canberra Packard, Berkshire, UK). Mean $\pm$ SEM counts per minute for triplicate cultures were expressed as a ratio of the Con A-treated and untreated PBL, i.e. a stimulation index.

4.2.10. HISTOLOGY

Upon termination, livers and pancreases were removed from animals and immediately fixed in neutral buffered formalin. In long-term surviving rats that received a 2° islet graft under the kidney capsule, the kidney was also removed and fixed. Representative sections of tissue were cut (4 μm) and stained as previously described.

4.2.11. STATISTICAL ANALYSIS

All results are presented as mean ± SEM and were analysed using unpaired Student's t-test. Differences between islet graft survival in Figure 10 were determined using the Mantel-Haenszel test.

4.3 RESULTS

4.3.1. Comparison of insulin secretion during static incubation of islets isolated from Wistar and Wistar Han rats.

Table 7 compares the insulin secretion (as % of TIC) from Wistar and WH rat islets in static incubation. All WH rat islets secreted significantly more insulin in basal glucose medium over a 48 hour culture period compared with Wistar rat islets ($p<0.01$ for 320 g and 370 g and $p<0.001$ for 280 g WH rat). Islets isolated from 320 g and 370 g WH rats secreted significantly less ($p<0.05$) insulin compared with islets isolated from 280 g WH rats. Following a 90 minute incubation in basal glucose medium, islets isolated from 280 g and 320 g WH rats secreted significantly more insulin ($p<0.01$ and $p<0.05$ respectively) compared with Wistar rat islets. Upon

Table 7. Comparison of insulin secretion (% of TIC) during static incubation of islets isolated from Wistar and Wistar Han rats.

Rat species	Rat weight (g)	INSULIN SECRETION (% OF TIC) Basal glucose (48 hours)	1° Basal glucose (90 min)	Stimulatory glucose (90 min)	2° Basal glucose (90 min)
Wistar	250	87.9 ± 5.6	0.5 ± 0	7.2 ± 1.0 ‡	4.1 ± 0.5 †,§§
Wistar Han	280	246.4 ± 22.7 ***	1.8 ± 0.2 **	10.6 ± 0.7 *,‡	2.1 ± 0.2 §
	320	155.6 ± 14.0 **, †	6.0 ± 1.5 *	12.4 ± 1.1 *,‡‡	3.8 ± 0.4 †,§
	370	163.3 ± 18.9 **, †	2.9 ± 1.9	10.4 ± 3.1	3.3 ± 0.2 ††

Mean ± SEM
Basal and stimulatory glucose concentration = 2.7 mmol/l and 16.7 mmol/l respectively.

* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared with Wistar rat.
† $p<0.05$ and †† $p<0.01$ compared with 280 g Wistar Han rat.
‡‡ $p<0.05$ and ‡ $p<0.001$ compared with 1° basal glucose.
§§ $p<0.05$ and § $p<0.001$ compared with stimulatory glucose.

replacement with high glucose medium, insulin secretion from Wistar and WH (280 g and 320 g) rat islets increased significantly ($p<0.001$, $p<0.001$ and $p<0.05$ respectively) and insulin secreted by islets isolated from 280 g and 320 g WH rats was significantly higher ($p<0.05$) compared with Wistar rat islets. Upon returning to basal glucose medium, insulin secretion from Wistar ($p<0.05$) and 280 g and 320 g WH ($p<0.001$) rat islets significantly decreased. The insulin secreted by islets isolated from 280 g WH rats was significantly lower than islets isolated from Wistar ($p<0.05$), 320 g ($p<0.05$) and 370 g ($p<0.01$) WH rats. Consequently, WH rats weighing between 280 g and 320 g were used for the isolation of islets in this study.

4.3.2. Comparison of the periods of normoglycaemia following intraportal islet transplantation in moAb-treated diabetic BB/E rats.

Table 8 shows the periods of normoglycaemia following a primary (1°) intraportal islet transplant in diabetic BB/E rats receiving depleting or non-depleting anti-CD4 or depleting anti-CD8 moAb treatment. Animals maintaining a long-term functional graft (79 ± 4 days) received a secondary (2°) islet transplant under the kidney capsule and were killed 31 ± 3 days later. Only one animal receiving a 2° islet graft was killed after returning to a hyperglycaemic state. Three animals were killed due to megacolon while still normoglycaemic. The periods of normoglycaemia following transplantation of 1° islet grafts were significantly greater for OX8, OX38 ($p<0.05$) and OX8/OX38 ($p<0.01$) moAb-treated rats compared with PBS-treated rats. Figure 10 shows the percentage of functioning islet grafts (i.e. grafts maintaining normoglycaemia) in diabetic BB/E rats following short-term (14 days) treatment with depleting or non-depleting anti-CD4 or depleting anti-CD8 moAb. OX8, OX38 and OX8/OX38 moAb-treated diabetic BB/E rats maintained functional islet grafts for significantly longer than PBS-treated control rats ($p<0.001$). MoAb treatments were

Table 8. Comparison of periods of normoglycaemia following intraportal islet transplantation in moAb-treated diabetic BB/E rats.

Recipient treatment	n	Days of normoglycaemia following intraportal islet transplantation	Mean ± SEM
None (PBS)	8	2, 3, 3, 3, 4, 6, 6, 6	4 ± 1
W3/25	9	2, 3, 4, 4, 5, 14, 15, 22,122 ‡	21 ± 13
OX8	9	8, 10, 15, 19, 21, 22, 23, 28 ¶, 95 §	27 ± 9 *
OX38	9	10, 10, 10, 13, 17, 34, 95 †, 98 ‡, 125 ‡	46 ± 16 *
OX8/OX38	9	8, 10, 10, 21, 25, 98 ‡, 110 ‡, 115 †, 125 ‡	58 ± 17 **

* $p<0.05$ and ** $p<0.01$ compared with untreated group.

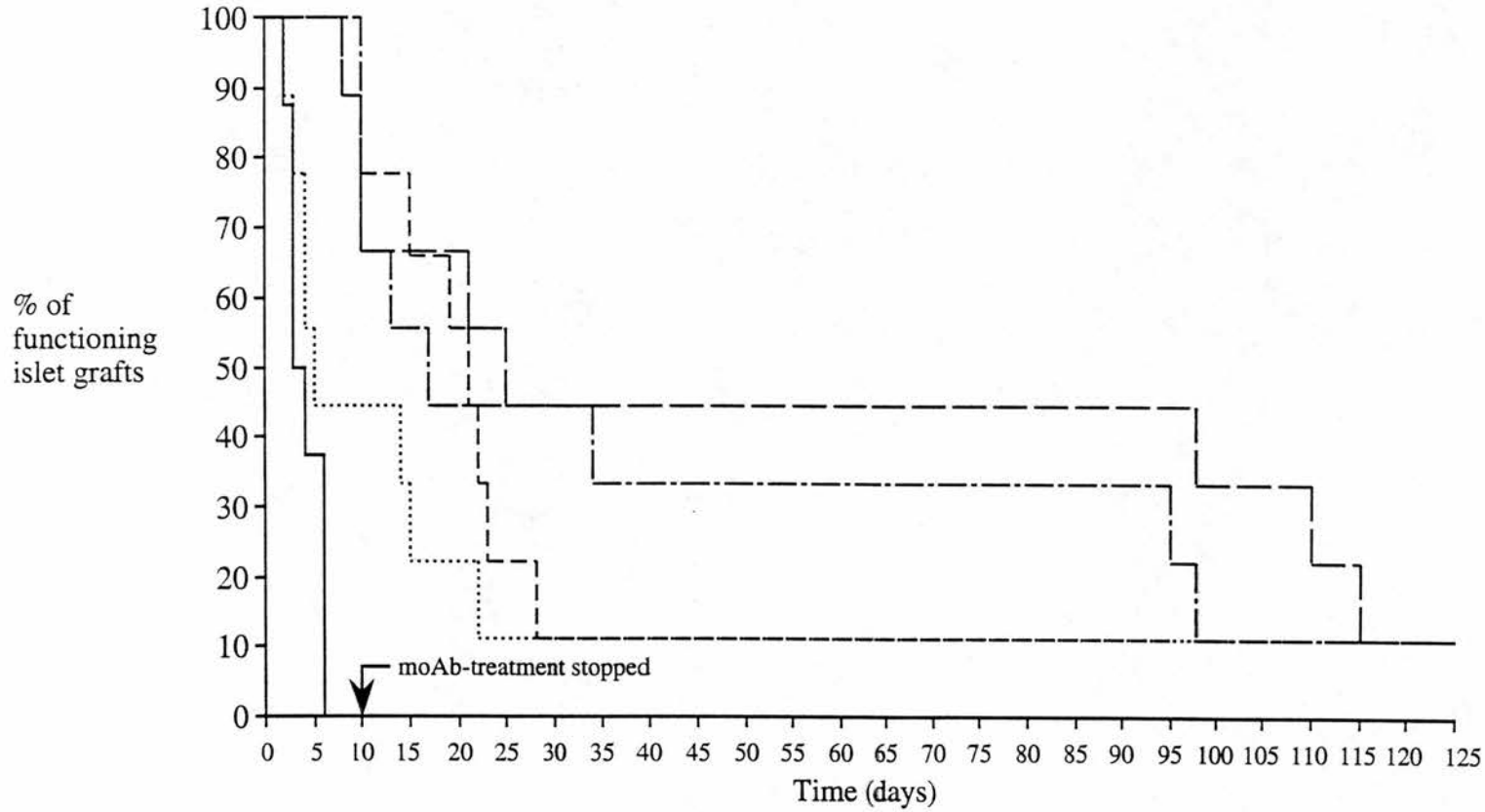
¶ killed due to megacolon while still normoglycaemic.

‡ recipient of a 2° islet graft under the kidney capsule and killed while still normoglycaemic.

† recipient of a 2° islet graft under the kidney capsule and killed due to megacolon while still normoglycaemic.

§ recipient of a 2° islet graft under the kidney capsule and killed upon return to hyperglycaemia.

Figure 10. Effect of moAb treatment on islet graft survival in diabetic BB/E rats.



Key:

——— PBS-treated rats (Controls)
····· W3/25 moAb-treated rats
– – – · OX8 moAb-treated rats
—·— OX38 moAb-treated rats
— — Combined OX8/OX38 moAb-treated rats

also compared using the χ^2 test for rectangular contingency tables after separating islet-transplanted BB/E rats into three groups (A, B and C) determined by the periods of normoglycaemia following an intraportal islet graft. Group A comprised animals with an intraportal islet graft that functioned for less than 10 days (end of PBS or moAb treatment) and therefore represented early graft failures. Group B comprised rats with a 1° islet graft that functioned ≥ 10 days (short-term functioning grafts). The diabetic BB/E rats in group C maintained long-term functioning 1° islet grafts and subsequently received a 2° islet graft under the kidney capsule on day 79 ± 4. The number of islet-transplanted BB/E rats receiving moAb treatment in each group is given in Table 9. Islet grafts survived for significantly longer in OX8, OX38 and OX8/OX38 moAb-treated diabetic rats compared with islet-transplanted control rats ($p<0.001$) and islets transplanted into OX38 moAb-treated rats survived for significantly longer than islets transplanted into W3/25 moAb-treated rats ($p<0.05$).

4.3.3. FACS analysis

Results of FACS analysis of PBL and lymphoid tissues are presented as both percentages and absolute cell numbers. Lymphocytes were expressed as % of total cells. Lymphocyte subsets were expressed as % of lymphocytes.

Table 9. Comparison of number of islet-transplanted diabetic BB/E rats receiving moAb treatment in groups A, B and C.†

Recipient treatment	n	Group A	Group B	Group C
None (PBS)	8	8	0	0
W3/25	9	5	3	1
OX8	9	1	7	1 *
OX38	9	0	6	3 *,**
OX8/OX38	9	1	4	4 *

† Diabetic BB/E rats were divided into three groups determined by the number of days of normoglycaemia observed following an intraportal islet graft. Group A : early graft failures (<10 days). Group B : short-term functioning grafts (≥10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

Statistical analysis of data was performed using χ^2 test for rectangular contingency tables.

* $p<0.001$ compared with PBS-treated control group.

** $p<0.05$ compared with W3/25 moAb-treated group.

4.3.3.1. Percentages

4.3.3.1.1. PBL and PBL subsets in PBS-treated or short-term moAb-treated diabetic BB/E rats.

The % lymphocytes and lymphocyte subsets in PBS-treated (control) or short-term W3/25, OX8, OX38 or OX8/OX38 moAb-treated diabetic BB/E rats are shown in Tables 10 - 14 respectively.

The % lymphocytes was significantly decreased 120 ($p<0.001$) and 240 minutes ($p<0.05$) and 24 hours ($p<0.01$) following the priming injection of OX8 moAb compared with pre-moAb treatment. The % lymphocytes was also significantly decreased during ($p<0.01$) and 1 week after the end of moAb treatment ($p<0.05$). The % lymphocytes staining for the cytotoxic/suppressor T cell marker, CD8 was significantly decreased 60 ($p<0.05$),120 and 240 minutes and 24 hours after the priming moAb injection and also during, at and 1 week after the end of moAb treatment compared with the value before moAb treatment ($p<0.01$). The % lymphocytes staining for both CD4 (helper) and CD8 T cell markers was significantly decreased 240 minutes and 24 hours following the priming injection of OX8 moAb and were completely depleted during moAb treatment compared with pre-moAb treatment ($p<0.05$).

The % $CD8^+$ lymphocytes was significantly reduced 120 and 240 minutes and 24 hours following the priming injection of OX38 moAb compared with the value prior to moAb treatment ($p<0.05$). The % of these cells was also significantly reduced during, at ($p<0.001$) and 1 week after ($p<0.05$) the end of moAb treatment The % $CD4^+$ lymphocytes was significantly reduced 24 hours following moAb injection

Table 10. PBL and PBL subsets (%) in PBS-treated diabetic BB/E rats.

	LYMPHOCYTES and LYMPHOCYTE SUBSETS (%)								
Lymphocyte subset†	Pre-moAb treatment (day -3)	+60 min	+120 min	+240 min	+24 hr (day -2)	During moAb treatment (day 0)	End of moAb treatment (day 10)	1 week post-moAb	2 weeks post-moAb (death)
lymphocytes	45.0 ± 2.6	33.3 ± 2.3	41.3 ± 4.3	40.3 ± 1.3	36.7 ± 6.1	40.3 ± 4.8	36.0 ± 2.5	19.7 ± 5.2	57.3 ± 5.2
$CD5^+$	5.7 ± 0.7	5.0 ± 0.6	4.3 ± 0.7	5.3 ± 0.9	4.3 ± 1.2	5.0 ± 0.6	6.0 ± 0	5.7 ± 0.9	2.7 ± 0.7
IgM^+	53.7 ± 9.5	34.7 ± 3.7	36.0 ± 0.6	31.3 ± 4.1	23.7 ± 7.0	46.3 ± 7.7	35.3 ± 3.4	55.3 ± 1.3	49.0 ± 7.8
$CD8^+$	12.3 ± 2.3	10.0 ± 1.5	10.7 ± 2.3	8.0 ± 1.7	10.0 ± 1.5	14.7 ± 3.0	10.7 ± 1.3	11.3 ± 1.5	21.7 ± 8.1
$CD4^+$	29.3 ± 2.3	37.3 ± 8.6	31.0 ± 4.0	35.3 ± 0.7	24.3 ± 9.7	25.0 ± 6.9	33.7 ± 4.4	32.7 ± 1.2	30.3 ± 9.0
$CD4^+/CD8^+$	7.0 ± 0.6	4.7 ± 0.7	4.7 ± 0.3	3.0 ± 0.6	3.0 ± 2.1	4.3 ± 2.3	6.7 ± 0.9	5.7 ± 1.9	3.3 ± 1.7

Mean ± SEM

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

Table 11. PBL and PBL subsets (%) in short-term W3/25 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES and LYMPHOCYTE SUBSETS (%)								
Lymphocyte subset†	Pre-moAb treatment (day -3)	+60 min	+120 min	+240 min	+24 hr (day -2)	During moAb treatment (day 0)	End of moAb treatment (day 10)	1 week post-moAb	2 weeks post-moAb (death)
lymphocytes	48.0 ± 4.2	37.7 ± 4.7	42.7 ± 0.9	41.0 ± 0.6	38.7 ± 1.7	37.7 ± 3.2	43.3 ± 0.7	29.3 ± 10.5	56.0 ± 15.5
$CD5^+$	4.7 ± 0.9	5.3 ± 1.3	4.3 ± 0.7	3.3 ± 0.7	4.7 ± 0.7	5.3 ± 0.7	5.7 ± 0.7	5.0 ± 0.6	3.0 ± 0.6
IgM^+	44.0 ± 4.7	35.3 ± 3.0	32.0 ± 4.9	28.3 ± 3.4	46.7 ± 5.8	46.3 ± 5.8	53.0 ± 13.2	50.7 ± 7.0	39.0 ± 4.5
$CD8^+$	9.0 ± 1.0	12.7 ± 1.3	10.7 ± 1.5	6.0 ± 0.3	12.0 ± 0.6	12.7 ± 0.3	14.3 ± 1.8	13.3 ± 1.9	23.3 ± 3.2
$CD4^+$	39.0 ± 2.3	29.0 ± 7.0	33.0 ± 5.7	45.0 ± 7.6	25.7 ± 9.2	29.3 ± 8.1	33.0 ± 2.1	37.7 ± 3.5	42.7 ± 6.2
$CD4^+/CD8^+$	9.7 ± 2.2	4.7 ± 0.7	7.7 ± 2.3	5.0 ± 0.6	5.0 ± 4.0	2.7 ± 1.8	10.7 ± 2.3	8.3 ± 4.2	6.3 ± 0.9

Mean ± SEM

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

Table 12. PBL and PBL subsets (%) in short-term OX8 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES and LYMPHOCYTE SUBSETS (%)								
Lymphocyte subset†	Pre-moAb treatment (day -3)	+60 min	+120 min	+240 min	+24 hr (day -2)	During moAb treatment (day 0)	End of moAb treatment (day 10)	1 week post-moAb	2 weeks post-moAb (death)
lymphocytes	44.3 ± 0.3	33.7 ± 5.7	35.7 ± 0.9[c]	24.7 ± 6.9[a]	26.0 ± 3.5[b]	33.3 ± 1.8[b]	35.3 ± 4.2	18.3 ± 7.3[a]	46.3 ± 4.9
$CD5^+$	5.3 ± 1.2	5.0 ± 1.2	4.7 ± 0.3	3.7 ± 0.9	6.3 ± 1.9	6.3 ± 1.9	6.0 ± 0.6	3.7 ± 0.3	3.7 ± 0.9
IgM^+	47.3 ± 7.1	28.7 ± 10.1	30.3 ± 2.9	24.0 ± 9.2	25.3 ± 13.4	49.0 ± 4.7	32.7 ± 2.3	58.0 ± 9.7	52.0 ± 6.5
$CD8^+$	10.3 ± 1.2	3.0 ± 1.5[a]	2.7 ± 0.3[b]	1.0 ± 0.6[b]	0.7 ± 0.3[b]	1.0 ± 1.0[b]	0.3 ± 0.3[b]	1.3 ± 0.9[b]	3.7 ± 3.2
$CD4^+$	34.0 ± 7.1	54.3 ± 7.6	29.7 ± 6.4	21.0 ± 1.5	29.3 ± 13.4	33.3 ± 7.3	39.7 ± 5.0	44.7 ± 2.7	36.7 ± 14.1
$CD4^+/CD8^+$	7.0 ± 2.1	6.3 ± 0.7	1.7 ± 0.3	1.0 ± 0.0[a]	0.7 ± 0.7[a]	0[a]	1.0 ± 1.0	1.3 ± 0.9	5.0 ± 3.5

Mean ± SEM

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ compared with pre-moAb treatment.

Table 13. PBL and PBL subsets (%) in short-term OX38 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES and LYMPHOCYTE SUBSETS (%)								
Lymphocyte subset†	Pre-moAb treatment (day -3)	+60 min	+120 min	+240 min	+24 hr (day -2)	During moAb treatment (day 0)	End of moAb treatment (day 10)	1 week post-moAb	2 weeks post-moAb (death)
lymphocytes	36.3 ± 3.5	31.7 ± 4.8	33.3 ± 3.2	27.7 ± 4.7	27.0 ± 5.3	34.0 ± 4.4	27.7 ± 10.5	24.0 ± 11.1	35.7 ± 5.0
$CD5^+$	7.0 ± 1.5	6.3 ± 3.4	6.3 ± 2.4	5.0 ± 2.1	7.7 ± 5.2	3.3 ± 0.3	15.7 ± 9.7	7.3 ± 1.5	3.7 ± 1.5
IgM^+	47.0 ± 5.5	33.0 ± 0.6	34.0 ± 3.1	23.7 ± 2.6	22.3 ± 11.1	47.3 ± 8.1	33.0 ± 1.2	50.3 ± 2.9	45.0 ± 4.0
$CD8^+$	11.7 ± 2.0	10.5 ± 2.1	5.7 ± 0.9[a]	2.7 ± 0.3[a]	1.7 ± 1.2[a]	0.3 ± 0.3[c]	0.7 ± 0.7[c]	2.7 ± 1.5[a]	14.3 ± 4.9
$CD4^+$	30.7 ± 3.4	42.0 ± 5.9	34.0 ± 3.1	32.3 ± 8.7	13.0 ± 6.7[d]	28.0 ± 9.0	37.7 ± 1.5	46.7 ± 7.2	49.0 ± 3.8
$CD4^+/CD8^+$	7.3 ± 0.9	5.0 ± 2.0	2.7 ± 1.2[a]	2.7 ± 0.9[a]	0.3 ± 0.3[b]	0.3 ± 0.3[b]	1.7 ± 0.9[a]	4.0 ± 2.6	9.3 ± 1.5

Mean ± SEM

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ compared with pre-moAb treatment.
[d] $p<0.05$ compared with value after 60 minutes.

Table 14. PBL and PBL subsets (%) in short-term combined OX8/OX38 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES and LYMPHOCYTE SUBSETS (%)								
Lymphocyte subset†	Pre-moAb treatment (day -3)	+60 min	+120 min	+240 min	+24 hr (day -2)	During moAb treatment (day 0)	End of moAb treatment (day 10)	1 week post-moAb	2 weeks post-moAb (death)
lymphocytes	48.3 ± 4.5	36.7 ± 7.9	36.3 ± 6.3	25.7 ± 2.0[a]	24.7 ± 5.3[a]	34.0 ± 4.2	40.3 ± 3.5	23.7 ± 6.4[a]	48.0 ± 3.0
$CD5^+$	7.3 ± 0.9	5.7 ± 2.2	5.0 ± 0.6	4.3 ± 1.5	6.0 ± 3.5	3.7 ± 0.3	5.0 ± 0	3.7 ± 0.9	2.7 ± 0.7
IgM^+	36.3 ± 1.9	31.7 ± 4.8	28.0 ± 1.5	20.7 ± 3.8	26.7 ± 14.4	46.7 ± 6.1	34.0 ± 1.5	50.7 ± 8.1	45.3 ± 7.7
$CD8^+$	10.0 ± 0.6[d]	6.3 ± 0.3[b]	3.7 ± 0.7[b]	1.0 ± 0.6[c]	1.0 ± 1.0[c]	0[c]	0.3 ± 0.3[c]	2.3 ± 2.3[b]	17.7 ± 1.9
$CD4^+$	37.3 ± 0.3	40.3 ± 9.3	32.7 ± 8.7	19.3 ± 2.6[b]	12.0 ± 8.3[a]	26.7 ± 11.8[b]	34.3 ± 4.7	43.7 ± 3.8	39.0 ± 2.5
$CD4^+/CD8^+$	9.3 ± 3.4	5.7 ± 4.2	2.7 ± 1.2	0.3 ± 0.3	0.3 ± 0.3	0	1.3 ± 0.9	4.0 ± 3.1	5.0 ± 1.0

Mean ± SEM

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ compared with pre-moAb treatment.
[d] $p<0.05$ compared with death.

compared with the value 60 minutes after injection ($p<0.05$). The % $CD4^+/CD8^+$ lymphocytes was significantly reduced 120 and 240 minutes ($p<0.05$) and 24 hours ($p<0.01$) following the priming injection of OX38 moAb compared with pre-moAb treatment. During and at the end of moAb treatment, the % of these cells was also significantly decreased ($p<0.01$ and $p<0.05$ respectively).

The % lymphocytes was significantly reduced 240 minutes and 24 hours following the priming injection of OX8/OX38 moAb and also 1 week post-moAb treatment compared with the value prior to moAb treatment ($p<0.05$). The % $CD8^+$ cells was significantly reduced 60, 120 ($p<0.01$) and 240 minutes ($p<0.001$) and 24 hours ($p<0.01$) following the priming moAb injection compared with pre-moAb treatment. The % of these cells was also significantly decreased during, at ($p<0.001$) and 1 week after ($p<0.01$) the end of moAb treatment. The % $CD4^+$ cells was significantly reduced 240 minutes ($p<0.001$) and 24 hours ($p<0.05$) following the priming injection of moAb compared with pre-moAb treatment. The % $CD4^+/CD8^+$ lymphocytes was significantly reduced 240 minutes ($p<0.01$) and 24 hours ($p<0.05$) following the priming injection of OX8/OX38 moAb and also during moAb treatment when $CD4^+/CD8^+$ lymphocytes were completely depleted compared with the value prior to moAb treatment ($p<0.01$).

The absolute effect of each moAb treatment was determined by comparison of the % lymphocytes and lymphocyte subsets of PBS-treated and moAb-treated rats.

The % $CD8^+$ lymphocytes was significantly reduced 60, 120, 240 minutes ($p<0.05$) and 24 hours ($p<0.01$) following moAb injection, during ($p<0.05$), at and 1 week after the end of moAb treatment ($p<0.01$) in OX8-treated diabetic BB/E rats. However, upon killing of OX8 moAb-treated animals 2 weeks post-moAb treatment,

the % $CD8^+$ T cells were not significantly different compared to pretreatment or to PBS-treated control rats. The % $CD4^+/CD8^+$ lymphocytes was significantly reduced in diabetic BB/E rats 120 and 240 minutes after the priming injection of OX8 moAb ($p<0.01$ and $p<0.05$ respectively) and at the end of treatment ($p<0.05$).

The % $CD8^+$ T cells was significantly reduced 240 minutes and 24 hours ($p<0.05$) after moAb treatment was started, during and at the end of moAb treatment ($p<0.01$) and 1 week post-moAb treatment ($p<0.05$) in OX38 moAb-treated rats compared with PBS-treated rats. At the time of killing there was no significant different in the % $CD8^+$ T cells compared with PBS-treated rats, although % lymphocytes was significantly higher in the control group ($p<0.05$). At the end of moAb treatment, % $CD4^+/CD8^+$ T lymphocytes was significantly reduced in OX38 moAb-treated diabetic BB/E rats ($p<0.05$).

The % lymphocytes were significantly reduced 240 minutes after the priming injection of OX8/OX38 moAb ($p<0.01$). The % $CD8^+$ T cells was significantly decreased 120 and 240 minutes ($p<0.05$) and 24 hours after moAb treatment was started and during, at and 1 week after the end of moAb treatment ($p<0.05$) compared with PBS-treated rats. Although the % $CD4^+$ T cells was significantly greater in OX8/OX38 moAb-treated BB/E rats prior to treatment ($p<0.05$), the % of these cells was significantly reduced 240 minutes following moAb injection ($p<0.01$). The % $CD4^+/CD8^+$ T cells was also significantly lowered in BB/E rats 240 minutes after priming injection of OX8/OX38 moAb and at the end of treatment ($p<0.05$) compared with PBS-treated rats.

4.3.3.1.2. The effect of PBS treatment or short-term moAb treatment on PBL and PBL subsets in islet-transplanted diabetic BB/E rats.

The % lymphocytes and lymphocyte subsets in islet-transplanted diabetic BB/E rats following PBS-treatment or short-term W3/25, OX8, OX38 or OX8/OX38 moAb treatment are shown in Tables 15 - 19 respectively. Lymphocyte data for diabetic BB/E rats was analysed after separating experimental animals into 3 groups depending upon the period of normoglycaemia following 1° islet transplantation on day 0 as previously described.

Islet-transplanted PBS-treated rats were all group A and had significantly increased % $CD4^+$ T cells at failure of the 1° islet graft compared with the % of these cells pretransplant and at death ($p<0.05$).

The % lymphocytes at the end of moAb treatment in group B was significantly lower in W3/25 moAb-treated BB/E rats receiving an intraportal islet graft compared with % lymphocytes pretreatment and pretransplant ($p<0.05$).

In OX8 moAb-treated rats, the % $CD8^+$ T cells was significantly decreased ($p<0.05$) prior to islet transplantation compared with pre-moAb treatment. $CD8^+$ T cells was significantly lower pretransplant ($p<0.01$), at the end of moAb treatment and upon islet graft failure ($p<0.05$) in group B compared with % $CD8^+$ T cells upon killing, although there was no significant difference between the % $CD8^+$ T lymphocytes at the time of death and prior to moAb treatment. The % $CD4^+$ T cells was significantly increased at failure of the intraportal islet graft compared with values at the end of treatment and upon killing ($p<0.01$). Upon combination of groups A, B and C, the % lymphocytes at the time of islet graft failure was significantly greater than at the

Table 15. PBL and PBL subsets (%) in islet-transplanted diabetic BB/E rats receiving PBS-treatment.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)					
Sub-group* (n)	Lymphocyte subset†	Pre-moAb treatment	Pretransplant of 1° islet graft	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
A (8)	lymphocytes	28.5 ± 3.4	38.1 ± 3.5	29.9 ± 3.7	28.5 ± 4.8	-	43.6 ± 5.4
	$CD5^+$	7.0 ± 0.9	3.1 ± 0.9	5.1 ± 0.7	6.8 ± 1.0	-	3.3 ± 0.3
	IgM^+	35.0 ± 4.5	57.8 ± 8.9	55.3 ± 8.5	53.2 ± 8.7		68.0 ± 5.2
	$CD8^+$	7.0 ± 0.7	6.1 ± 2.6	7.7 ± 0.9	6.0 ± 1.5	-	17.0 ± 3.9
	$CD4^+$	36.4 ± 5.6	26.0 ± 5.9[a]	43.0 ± 2.5	32.7 ± 7.0	-	34.7 ± 2.8[a]
	$CD4^+/CD8^+$	2.9 ± 1.0	1.1 ± 0.4	3.8 ± 0.9	5.8 ± 2.9	-	11.0 ± 2.0

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

[a] $p<0.05$ compared with failure of 1° islet graft.

Table 16. The effect of short-term W3/25 moAb treatment on PBL and PBL subsets (%) in islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)					
Sub-group* (n)	Lymphocyte subset†	Pre-moAb treatment‡	Pretransplant of 1° islet graft‡	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
A (5)	lymphocytes	34.8 ± 4.2	35.4 ± 4.8	24.3 ± 6.8	28.2 ± 8.5	-	45.0 ± 6.8
	$CD5^+$	4.1 ± 0.4	3.8 ± 0.4	6.5 ± 2.5	6.4 ± 2.2	-	2.4 ± 0.4
	IgM^+	52.9 ± 6.7	57.0 ± 6.4	74.0 ± 8.5	65.6 ± 5.1	-	57.2 ± 6.2
	$CD8^+$	6.6 ± 1.5	9.3 ± 2.9	2.8 ± 1.1	6.8 ± 2.3	-	17.8 ± 4.6
	$CD4^+$	38.7 ± 5.4	33.7 ± 4.3	31.8 ± 6.7	25.2 ± 6.8	-	30.2 ± 4.4
	$CD4^+/CD8^+$	2.6 ± 0.9	3.0 ± 1.2	4.3 ± 1.9	8.0 ± 5.1	-	15.6 ± 5.0
B (3)	lymphocytes	-	-	8.0	$20.5 \pm 0.5^{a,b}$	-	33.0 ± 6.4
	$CD5^+$	-	-	3.0	3.0 ± 0.0	-	4.0 ± 0.6
	IgM^+	-	-	30.0	34.5 ± 16.5	-	57.3 ± 10.8
	$CD8^+$	-	-	1.0	6.0 ± 2.0	-	9.0 ± 1.5
	$CD4^+$	-	-	68.0	48.0 ± 17.0	-	37.0 ± 6.8
	$CD4^+/CD8^+$	-	-	3.0	5.0 ± 1.0	-	5.3 ± 0.2

C (1)	lymphocytes	-	-	-	29.0	43.0	50.0
	$CD5^+$	-	-	-	4.0	3.0	1.0
	IgM^+	-	-	-	42.0	37.0	53.0
	$CD8^+$	-	-	-	6.0	8.0	32.0
	$CD4^+$	-	-	-	36.0	48.0	29.0
	$CD4^+/CD8^+$	-	-	-	2.0	3.0	3.0
TOTAL (9)	lymphocytes	-	-	21.0 ± 6.2	26.4 ± 5.3	43.0	41.6 ± 4.6
	$CD5^+$	-	-	5.8 ± 2.1	5.3 ± 1.4	3.0	2.8 ± 0.4
	IgM^+	-	-	65.2 ± 11.0	54.9 ± 6.9	37.0	56.8 ± 4.6
	$CD8^+$	-	-	2.4 ± 0.9	6.5 ± 1.5	8.0	16.4 ± 3.4
	$CD4^+$	-	-	39.0 ± 8.9	32.3 ± 6.4	48.0	32.3 ± 3.9
	$CD4^+/CD8^+$	-	-	4.0 ± 1.5	6.5 ± 3.2	3.0	10.8 ± 3.3

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

‡ The data for pre-moAb treatment and pretransplant of the 1° islet graft is expressed as a total for groups A, B and C.

[a] $p<0.05$ compared with pre-moAb treatment.
[b] $p<0.05$ compared with pretransplant of 1° islet graft.

Table 17. The effect of short-term OX8 moAb treatment on PBL and PBL subsets (%) in islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)					
Sub-group* (n)	Lymphocyte subset†	Pre-moAb treatment‡	Pretransplant of 1° islet graft‡	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
A (1)	lymphocytes	24.3 ± 3.7	25.9 ± 4.5	36.0	13.0	-	41.0
	$CD5^+$	6.6 ± 1.0	5.0 ± 0.7	4.0	4.0	-	2.0
	IgM^+	45.9 ± 6.8	59.0 ± 6.9	48.0	31.0	-	81.0
	$CD8^+$	4.0 ± 1.3	0.2 ± 0.2[a,b,c,d]	0.0	0.0	-	6.0
	$CD4^+$	42.4 ± 4.9[e]	29.6 ± 4.6	39.0	74.0	-	45.0
	$CD4^+/CD8^+$	2.4 ± 1.0	0.4 ± 0.2	1.0	0.0	-	10.0
B (7)	lymphocytes	-	-	36.0 ± 5.4	21.7 ± 5.0	-	34.1 ± 6.9
	$CD5^+$	-	-	5.3 ± 1.1	7.2 ± 1.2	-	5.7 ± 2.6
	IgM^+	-	-	69.0 ± 16.2	49.5 ± 5.5	-	47.1 ± 7.3
	$CD8^+$	-	-	1.0 ± 1.0[f]	1.0 ± 0.7[f]	-	9.0 ± 2.7
	$CD4^+$	-	-	51.3 ± 2.5	34.0 ± 4.9[e]	-	34.3 ± 3.7[e]
	$CD4^+/CD8^+$	-	-	2.0 ± 1.4	1.3 ± 0.6	-	5.1 ± 2.2

C (1)	lymphocytes	-	-	-	31.0	40.0	57.0
	$CD5^+$	-	-	-	5.0	5.0	0.0
	IgM^+	-	-	-	38.0	36.0	0.0
	$CD8^+$	-	-	-	0.0	3.0	0.0
	$CD4^+$	-	-	-	33.0	52.0	0.0
	$CD4^+/CD8^+$	-	-	-	0.0	5.0	0.0
TOTAL (9)	lymphocytes	-	-	36.0 ± 4.2	21.8 ± 4.0[g]	40.0	37.4 ± 5.9
	$CD5^+$	-	-	5.0 ± 0.9	6.5 ± 1.0	5.0	4.7 ± 2.1
	IgM^+	-	-	64.8 ± 13.2	45.8 ± 4.8	36.0	51.4 ± 7.2
	$CD8^+$	-	-	0.8 ± 0.8[f]	0.8 ± 0.5[a,f]	3.0	7.7 ± 2.3
	$CD4^+$	-	-	48.8 ± 3.1	38.9 ± 6.2	52.0	35.6 ± 3.3[g]
	$CD4^+/CD8^+$	-	-	1.8 ± 1.1	1.0 ± 0.4	5.0	5.1 ± 1.9[h]

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.
‡ The data for pre-moAb treatment and pretransplant of the 1° islet graft is expressed as a total for groups A, B and C.

[a] $p<0.05$ compared with pre-moAb treatment.
[b] $p<0.01$ compared with death in group B.
[c] $p<0.01$ compared with death (TOTAL).
[d] $p<0.01$ compared with failure of 1° islet graft (TOTAL)
[e] $p<0.01$ compared with failure of 1° islet graft in group B.
[f] $p<0.05$ compared with death.
[g] $p<0.05$ compared with failure of 1° islet graft.
[h] $p<0.05$ compared with pretransplant of 1° islet graft.

Table 18. The effect of short-term OX38 moAb treatment on PBL and PBL subsets (%) in islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)					
Sub-group* (n)	Lymphocyte subset†	Pre-moAb treatment‡	Pretransplant of 1° islet graft‡	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
B (6)	lymphocytes	29.4 ± 5.0[a]	28.8 ± 4.8[a]	23.8 ± 5.5	29.0 ± 3.4	-	35.2 ± 5.1
	$CD5^+$	4.6 ± 0.5	2.6 ± 0.6[b,c]	5.8 ± 0.8	5.6 ± 0.5	-	2.7 ± 0.8
	IgM^+	43.6 ± 8.1	60.4 ± 7.9	46.5 ± 14.5	50.0 ± 13.4	-	59.8 ± 5.7
	$CD8^+$	4.3 ± 1.2[d,e]	2.4 ± 1.9[d,e]	1.8 ± 0.8[d]	1.2 ± 0.5[b,d]	-	14.5 ± 2.0
	$CD4^+$	41.1 ± 5.5	29.9 ± 6.7	48.0 ± 6.0	38.4 ± 4.9	-	36.0 ± 4.4
	$CD4^+/CD8^+$	2.9 ± 1.1[f,g]	1.3 ± 1.0[e,h]	1.8 ± 0.9[i]	2.6 ± 1.6	-	7.8 ± 1.9
C (3)	lymphocytes	-	-	-	13.3 ± 7.9[a]	47.7 ± 4.6	27.3 ± 7.8
	$CD5^+$	-	-	-	6.0 ± 2.1	4.0 ± 0.6	3.0 ± 1.5
	IgM^+	-	-	-	43.0 ± 6.1	50.7 ± 4.1	78.5 ± 0.5
	$CD8^+$	-	-	-	5.7 ± 5.2	4.0 ± 1.0	6.0 ± 1.0
	$CD4^+$	-	-	-	34.7 ± 2.7[i]	35.0 ± 12.5	44.5 ± 0.5
	$CD4^+/CD8^+$	-	-	-	2.7 ± 2.2	6.3 ± 2.8	5.0 ± 0.0

TOTAL (9)	lymphocytes	-	-	23.8 ± 5.5	23.1 ± 4.4	47.7 ± 4.6	32.6 ± 4.2
	$CD5^+$	-	-	5.8 ± 0.8	5.8 ± 0.8	4.0 ± 0.6	2.8 ± 0.7[j]
	IgM^+	-	-	46.5 ± 14.5	47.4 ± 8.4	50.7 ± 4.1	64.5 ± 5.2
	$CD8^+$	-	-	1.8 ± 0.8[k]	2.9 ± 1.9[e]	4.0 ± 1.0[e]	12.4 ± 2.0
	$CD4^+$	-	-	48.0 ± 6.0	37.0 ± 3.1	35.0 ± 12.5	38.9 ± 2.1
	$CD4^+/CD8^+$	-	-	1.8 ± 0.9[i]	2.6 ± 1.2[i]	6.3 ± 2.8	7.1 ± 1.5

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.
‡ The data for pre-moAb treatment and pretransplant of the 1° islet graft is expressed as a total for groups B and C.

[a] $p<0.05$ compared with pretransplant of 2° islet graft in group C.
[b] $p<0.05$ compared with pre-moAb treatment.
[c] $p<0.01$ compared with failure of 1° islet graft (TOTAL).
[f] $p<0.05$, [h] $p<0.01$, [d] $p<0.001$ compared with death in group B.
[g] $p<0.05$, [e] $p<0.01$ and [k] $p<0.001$ compared with death (TOTAL).
[i] $p<0.05$ compared with death.
[j] $p<0.05$ compared with failure of 1° islet graft.

Table 19. The effect of short-term combined OX8/OX38 moAb treatment on PBL and PBL subsets (%) in islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)					
Sub-group* (n)	Lymphocyte subset†	Pre-moAb treatment‡	Pretransplant of 1° islet graft‡	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
A (1)	lymphocytes	32.6 ± 3.8[a]	30.2 ± 3.4[b]	33.0	14.0	-	64.0
	$CD5^+$	5.3 ± 0.5	4.7 ± 0.8	8.0	9.0	-	2.0
	IgM^+	39.8 ± 7.8	50.6 ± 6.6	56.0	47.0	-	48.0
	$CD8^+$	7.0 ± 1.0[c,d]	0.4 ± 0.4[e,f,g]	1.0	0.0	-	24.0
	$CD4^+$	43.0 ± 3.5	39.7 ± 4.5	42.0	51.0	-	49.0
	$CD4^+/CD8^+$	4.6 ± 0.1	0.1 ± 0.1[d,e,h,i,j]	6.0	0.0	-	15.0
B (4)	lymphocytes	-	-	26.0 ± 1.0	23.7 ± 3.7	-	41.7 ± 10.7
	$CD5^+$	-	-	7.0 ± 2.0	5.3 ± 0.3	-	3.3 ± 0.3
	IgM^+	-	-	51.0 ± 11.0	72.0 ± 13.2	-	49.0 ± 9.8
	$CD8^+$	-	-	0[k]	0[k]	-	18.3 ± 4.6
	$CD4^+$	-	-	43.0 ± 4.0	42.3 ± 2.7	-	32.7 ± 8.8
	$CD4^+/CD8^+$	-	-	0	1.3 ± 0.9	-	3.3 ± 1.8

C (4)	lymphocytes	-	-	-	21.5 ± 2.4[a,l]	47.5 ± 3.8	35.5 ± 3.4
	$CD5^+$	-	-	-	5.5 ± 0.5	3.8 ± 0.8	3.8 ± 0.5
	IgM^+	-	-	-	36.3 ± 5.8	47.8 ± 5.0	69.8 ± 6.4
	$CD8^+$	-	-	-	1.8 ± 0.9[m]	5.0 ± 1.6	9.8 ± 1.7
	$CD4^+$	-	-	-	38.8 ± 5.5	29.0 ± 9.6	46.5 ± 1.0
	$CD4^+/CD8^+$	-	-	-	0.8 ± 0.5[b,e,m]	7.3 ± 1.3	7.8 ± 3.0
TOTAL (9)	lymphocytes	-	-	28.3 ± 2.4	21.4 ± 2.0[l,n]	47.5 ± 3.8	41.4 ± 5.1
	$CD5^+$	-	-	7.0 ± 1.2	5.9 ± 0.5	3.8 ± 0.8	3.4 ± 0.3
	IgM^+	-	-	52.7 ± 6.6	51.0 ± 8.1	47.8 ± 5.0	59.3 ± 5.9
	$CD8^+$	-	-	0.3 ± 0.3[e,g]	0.9 ± 0.5[e,g]	5.0 ± 1.6[o]	14.8 ± 2.6
	$CD4^+$	-	-	42.7 ± 2.3	41.6 ± 3.1	29.0 ± 9.6	42.8 ± 3.1
	$CD4^+/CD8^+$	-	-	2.0 ± 2.0[j]	0.9 ± 0.4[d,j,m]	7.3 ± 1.3	11.1 ± 4.5

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

‡ The data for pre-moAb treatment and pretransplant of the 1° islet graft is expressed as a total for groups A, B and C.

[a] $p<0.05$, [b] $p<0.01$ and [h] $p<0.001$ compared with pretransplant of 2° islet graft in group C.
[c] $p<0.05$ and [f] $p<0.01$ compared with death in group B
[d] $p<0.05$, [o] $p<0.01$ and [g] $p<0.001$ compared with death (TOTAL).
[l] $p<0.05$, [m] $p<0.01$ and [e] $p<0.001$ compared with pre-moAb treament.
[i] $p<0.05$ compared with death in group C.
[j] $p<0.001$ compared with pretransplant of 2° islet graft (TOTAL).
[k] $p<0.05$ compared with death.
[n] $p<0.05$ compared with pretransplant of 1° islet graft.

end of moAb treatment ($p<0.05$). The % $CD8^+$ T cells was significantly decreased prior to transplantion of the 1° islet graft ($p<0.01$), failure of the 1° graft and at the end of moAb treatment ($p<0.05$) compared with % $CD8^+$ T cells upon killing. There was no significant difference in % $CD8^+$ T cells between pre-moAb treatment and death. The % $CD4^+$ T cells was significantly greater upon failure of the 1° islet graft compared with cells prior to 1° islet transplantation and killing ($p<0.01$ and $p<0.05$ respectively). The % $CD4^+/CD8^+$ T cells was significantly greater upon killing compared with pretransplant of the 1° islet graft ($p<0.05$).

The % $CD5^+$ T cells was significantly decreased pretransplant of the intraportal graft in OX38 moAb-treated diabetic BB/E rats compared with pre-moAb treatment ($p<0.05$). However, there was no significant difference between the % $CD5^+$ T cells at failure of the 1° islet graft and the end of moAb treatment when compared with pre-moAb treatment in group B experimental rats. There was a significant decrease in the % $CD8^+$ T cells between pre-moAb treatment and the end of moAb treatment ($p<0.05$), and the % $CD8^+$ T cells upon killing was significantly higher than % of cells pre-moAb treatment, pretransplant, at failure of the 1° islet graft and the end of moAb treatment ($p<0.001$). The % $CD4^+/CD8^+$ T cells was also significantly increased at death compared with pre-moAb treatment ($p<0.05$), pretransplant ($p< 0.01$) and failure of the intraportal islet graft ($p<0.05$). In group C, the % lymphocytes was significantly increased at the time of the 2° islet graft under the kidney capsule compared with pre-moAb treatment, pretransplant of the 1° intraportal islet graft and at the end of moAb treatment ($p<0.05$). The % $CD4^+$ T cells significantly increased from the end of moAb treatment to the time of death ($p<0.05$). For the combined data of groups B and C, % $CD5^+$ T cells at failure of the 1° islet graft was significantly greater than the % of cells pretransplant and upon killing ($p<0.01$ and $p<0.05$ respectively). The % $CD8^+$ T cells was higher at killing

compared with pre-moAb treatment, pretransplant of both 1° and 2° islet grafts, end of moAb treatment ($p<0.01$) and failure of the 1° islet graft ($p<0.001$) in the OX38 moAb-treated rats. The % $CD4^+/CD8^+$ T cells was also significantly higher at death compared with pre-moAb treatment ($p<0.05$), pretransplant ($p<0.01$) and failure of the 1° graft and at the end of OX38 moAb treatment ($p<0.05$).

The % $CD8^+$ and $CD4^+/CD8^+$ T cells were both significantly reduced prior to transplantion of the intraportal islet graft in OX8/OX38 moAb-treated diabetic BB/E rats compared with pre-moAb treatment ($p<0.001$). In group B, % $CD8^+$ T cells upon killing of the BB/E rat was significantly higher compared with % of cells pre-moAb treatment ($p< 0.05$), pretransplant ($p<0.01$), failure of the 1° islet graft and at the end of moAb treatment ($p<0.05$). At these two latter time points, all $CD8^+$ T cells were completely depleted. The % $CD4^+/CD8^+$ cells was significantly reduced at the end of moAb treatment and were completely depleted at failure of the intraportal islet graft. The % lymphocytes decreased from pre-moAb treatment to the end of treatment in group C ($p<0.05$) and was significantly increased at the time of 2° islet grafting under the kidney capsule compared with values prior to moAb treatment ($p<0.05$), pretransplant ($p<0.01$) and end of treatment ($p<0.05$). The % $CD8^+$ T cells was significantly decreased at the end of treatment, pretransplant of the 2° islet graft and at death compared with pre-moAb treatment ($p<0.01$), but not at the time of failure of the 1° islet graft. The % $CD4^+/CD8^+$ T cells was significantly decreased from pre-moAb treatment to the end of moAb treatment ($p<0.01$) which was significantly lower compared with pretransplant of the 2° islet graft under the kidney capsule and at death ($p<0.01$ and $p<0.05$ respectively). The % of these cells was also significantly reduced prior to transplantation of the 1° islet graft compared with pretransplantation of the 2° islet graft ($p<0.001$) and at death ($p<0.05$). Upon combining the data for groups A, B and C, % lymphocytes before moAb treatment

and pretransplant were significantly higher compared with % lymphocytes at the end of OX8/OX38 moAb treatment ($p<0.05$), although this difference was not significant at death. The % $CD8^+$ T cells was significantly reduced at the time of 1° graft failure and the end of moAb treatment ($p<0.001$) but not prior to transplantation of the 2° islet graft, compared with pre-moAb treatment. However, upon killing, the % $CD8^+$ T cells was significantly higher than at pre-moAb treatment ($p<0.05$), pretransplant of the 1° ($p<0.001$) and 2° ($p<0.01$) islet grafts, failure of the 1° graft and at the end of OX8/OX38 moAb treatment ($p<0.001$). The % $CD4^+/CD8^+$ T cells was significantly reduced at the end of moAb treatment compared with that pre-moAb treatment ($p<0.01$). These cells were significantly higher prior to transplantation of the 2° islet graft under the kidney capsule compared with pretransplant and failure of the 1° islet graft and at the end of moAb treatment ($p<0.001$). Upon killing, the % $CD4^+/CD8^+$ T cells was significantly higher than at pretransplant and the end of treatment ($p<0.05$) but was not significantly different compared with the value prior to moAb treatment.

The % lymphocytes and lymphocyte subsets of PBS-treated diabetic BB/E rats receiving an intraportal islet graft (Table 15) were compared with islet-transplanted diabetic rats receiving W3/25, OX8, OX38 or OX8/OX38 moAb treatment (Tables 16 - 19).

Prior to intraportal islet transplantation, both the % lymphocytes and $CD8^+$ T cells were significantly lower in diabetic rats receiving OX8 moAb-treatment ($p<0.05$). The % $CD8^+$ T cells remained significantly reduced in group B at failure of the 1° graft and at the end of treatment compared with islet-transplanted rats receiving PBS treatment ($p<0.001$ and $p<0.05$ respectively). The % $CD4^+$ T cells was significantly increased at the time of failure of the 1° islet graft in islet-transplanted diabetic rats

receiving OX8 moAb-treatment ($p<0.05$). Combining the data for the groups A, B and C confirmed that the % $CD8^+$ T cells was significantly decreased in BB/E rats at the time of 1° islet graft failure and at the end of OX8 moAb treatment ($p<0.001$ and $p<0.01$ respectively).

The % $CD8^+$ T cells was significantly reduced in islet-transplanted diabetic rats receiving OX38 moAb-treatment in group B upon failure of the intraportal islet graft and at the end of moAb treatment compared with PBS-treated rats ($p<0.001$ and $p<0.05$ respectively). Upon killing of OX38 moAb-treated rats in group C, the % $CD8^+$ and $CD4^+/CD8^+$ T cells were significantly reduced ($p<0.05$).

The % $CD8^+$ and $CD4^+/CD8^+$ T cells were both significantly reduced in islet-transplanted diabetic rats receiving OX8/OX38 moAb-treatment at pretransplant compared with PBS-treated rats ($p<0.05$). The % of both lymphocyte subsets remained significantly reduced at the time of 1° islet graft failure in Group B ($p<0.001$ and $p<0.01$ respectively), and with respect to the % $CD8^+$ T cells, at the end of moAb treatment also ($p<0.01$). Upon killing, % $CD4^+/CD8^+$ T cells was significantly reduced ($p<0.05$). In group C, % $CD8^+$ T cells at the end of OX8/OX38 moAb-treatment was significantly reduced compared with PBS-treated rats ($p<0.05$), and were significantly reduced upon failure of the 1° islet graft and at the end of moAb treatment ($p<0.001$) upon combining the data from groups A, B and C.

To determine the effect of islet transplantation on % lymphocytes and lymphocyte subsets, PBS-treated (Table 10) or W3/25, OX8, OX38 or combined OX8/OX38 moAb-treated diabetic BB/E rats (Tables 11 - 14 respectively) were compared with the % cells in corresponding islet-transplanted rats receiving PBS-treatment or

W3/25, OX8, OX38 or combined OX8/OX38 moAb-treatment (Tables 16 - 19 respectively).

At the end of OX8 moAb treatment in group B, the % cells staining for the B lymphocyte marker, IgM^+ was significantly greater in islet-transplanted rats compared with rats treated with OX8 moAb alone ($p<0.05$). This finding was also observed when the data for groups A, B and C were combined.

The % IgM^+ B cells was significantly greater in islet-transplanted diabetic BB/E rats receiving OX38 moAb treatment at death in group C and upon combining the data for groups A, B and C ($p<0.01$ and $p<0.05$ respectively).

At the end of OX8/OX38 moAb treatment, diabetic BB/E rats in group B receiving an islet graft had significantly increased IgM^+ B cells ($p<0.05$) and % $CD4^+$ T cells was significantly increased ($p<0.05$) upon killing of rats in group C compared with OX8/OX38 moAb-treated rats .

4.3.3.1.3 Lymphocytes and lymphocyte subsets in lymphoid tissues of PBS-treated or short-term moAb-treated diabetic BB/E rats.

The % lymphocytes and lymphocyte subsets in the lymphoid tissues upon killing the PBS-treated or W3/25, OX8, OX38 or OX8/OX38 moAb-treated diabetic BB/E rats are shown in Tables (20 - 24). MoAb treatment had no effect on the % lymphocytes or lymphocyte subsets in the lymphoid tissues compared with PBS-treated rats.

Table 20. Lymphocyte and lymphocyte subsets (%) in lymphoid tissues of PBS-treated (control) diabetic BB/E rats.

	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)				
Lymphocyte subset †	PLN	MLN	Spleen	PEC	Thymus
Lymphocytes	75.7 ± 4.7	63.7 ± 18.4	59.7 ± 3.4	41.7 ± 4.9	95.3 ± 1.2
$CD5^+$	14.7 ± 2.4	5.7 ± 2.0	6.0 ± 1.5	2.3 ± 1.2	78.3 ± 7.7
IgM^+	20.3 ± 7.9	50.7 ± 16.4	52.7 ± 5.7	34.0 ± 23.1	1.0 ± 0.0
$CD8^+$	2.7 ± 0.3	1.3 ± 0.3	10.7 ± 3.7	5.3 ± 1.2	17.7 ± 5.9
$CD4^+$	16.7 ± 1.7	10.3 ± 2.4	15.0 ± 0.6	62.0 ± 17.5	5.7 ± 1.2
$CD4^+/CD8^+$	1.0 ± 1.0	2.0 ± 1.5	4.3 ± 3.0	20.7 ± 20.7	64.3 ± 15.1

Mean ± SEM. n = 3.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

Table 21. Lymphocyte and lymphocyte subsets (%) in lymphoid tissues of short-term W3/25 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)				
Lymphocyte subset †	PLN	MLN	Spleen	PEC	Thymus
Lymphocytes	72.0 ± 6.7	65.7 ± 10.4	41.0 ± 6.1	49.7 ± 9.5	95.3 ± 1.2
$CD5^+$	11.0 ± 1.2	4.3 ± 0.3	3.7 ± 1.2	0.7 ± 0.3	73.7 ± 11.0
IgM^+	11.7 ± 2.4	50.0 ± 11.7	45.3 ± 15.2	17.0 ± 9.2	4.0 ± 1.5
$CD8^+$	2.0 ± 0.0	1.0 ± 0.0	7.0 ± 1.0	3.7 ± 0.7	9.0 ± 1.5
$CD4^+$	15.7 ± 2.2	10.0 ± 1.5	13.0 ± 1.5	42.0 ± 26.1	5.7 ± 1.2
$CD4^+/CD8^+$	1.7 ± 0.7	1.3 ± 0.3	3.3 ± 1.9	0.3 ± 0.3	80.7 ± 3.2

Mean ± SEM. n = 3.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

Table 22. Lymphocyte and lymphocyte subsets (%) in lymphoid tissues of short-term OX8 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)				
Lymphocyte subset †	PLN	MLN	Spleen	PEC	Thymus
Lymphocytes	59.0 ± 13.0	67.3 ± 10.0	49.2 ± 4.8	49.7 ± 7.2	94.0 ± 1.2
$CD5^+$	11.7 ± 3.8	5.0 ± 1.2	4.3 ± 0.7	2.3 ± 0.9	77.3 ± 6.7
IgM^+	26.3 ± 13.6	40.7 ± 10.0	57.3 ± 1.2	25.3 ± 15.4	2.0 ± 0.6
$CD8^+$	1.3 ± 0.9	0.7 ± 0.3	3.7 ± 2.0	1.3 ± 0.9	8.7 ± 0.3
$CD4^+$	17.7 ± 4.4	9.7 ± 1.2	12.3 ± 1.5	67.7 ± 26.3	4.7 ± 1.2
$CD4^+/CD8^+$	3.3 ± 1.8	1.3 ± 0.7	5.0 ± 2.3	0	83.0 ± 1.5

Mean ± SEM. n = 3.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

Table 23. Lymphocyte and lymphocyte subsets (%) in lymphoid tissues of short-term OX38 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)				
Lymphocyte subset †	PLN	MLN	Spleen	PEC	Thymus
Lymphocytes	67.3 ± 5.8	68.3 ± 7.4	55.0 ± 3.1	43.0 ± 7.2	95.7 ± 0.3
$CD5^+$	14.7 ± 1.2	6.7 ± 1.5	5.3 ± 1.2	7.0 ± 2.6	83.0 ± 2.3
IgM^+	14.0 ± 9.0	37.0 ± 15.6	52.0 ± 3.2	30.0 ± 15.6	1.0 ± 0.0
$CD8^+$	2.3 ± 0.3	0.7 ± 0.3	6.7 ± 1.2	6.0 ± 2.0	8.3 ± 0.7
$CD4^+$	17.7 ± 1.9	10.3 ± 0.3	20.3 ± 5.9	61.7 ± 9.5	6.7 ± 0.3
$CD4^+/CD8^+$	1.0 ± 0.0	1.0 ± 0.0	4.3 ± 1.5	9.0 ± 5.5	81.3 ± 0.9

Mean ± SEM. n = 3.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

Table 24. Lymphocyte and lymphocyte subsets subsets (%) in lymphoid tissues of short-term combined OX8/OX38 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)				
Lymphocyte subset †	PLN	MLN	Spleen	PEC	Thymus
Lymphocytes	71.3 ± 9.3	76.7 ± 3.5	39.0 ± 12.3	52.0 ± 4.5	96.7 ± 0.3
$CD5^+$	13.0 ± 1.2	6.3 ± 0.9	3.3 ± 1.3	3.0 ± 1.2	80.7 ± 3.5
IgM^+	9.3 ± 2.0	33.3 ± 5.2	54.0 ± 5.7	42.0 ± 8.1	2.0 ± 1.0
$CD8^+$	2.0 ± 0.0	1.0 ± 0.0	6.0 ± 0.6	2.7 ± 0.3	9.0 ± 2.1
$CD4^+$	15.0 ± 1.5	9.7 ± 1.5	13.0 ± 4.5	53.7 ± 18.3	5.3 ± 1.3
$CD4^+/CD8^+$	2.0 ± 1.2	1.0 ± 0.0	6.3 ± 1.5	16.3 ± 10.5	83.0 ± 3.1

Mean ± SEM. n = 3.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

4.3.3.1.4. The effect of PBS treatment or short-term moAb treatment on lymphocytes and lymphocyte subsets in lymphoid tissues of islet-transplanted diabetic BB/E rats.

The % lymphocytes and lymphocyte subsets in the lymphoid tissues of islet-transplanted diabetic BB/E rats receiving PBS treatment or W3/25, OX8, OX38 or OX8/OX38 moAb treatment are shown in Tables 25 - 29 respectively. MoAb treatment had no effect on the % lymphocytes or lymphocyte subsets in the lymphoid tissues compared with PBS-treated rats.

The only finding of significance upon comparing the % lymphocytes and lymphocyte subsets of the lymphoid tissues of PBS-treated (Table 25) or W3/25, OX8, OX38 or OX8/OX38 moAb-treated (Tables 26 - 29) diabetic BB/E rats was the reduction in % $CD8^+$ T cells in the thymus of group B rats receiving OX8 moAb treatment at death ($p<0.05$). However, this reduction was not significant upon combining the data from groups A, B and C.

To determine the effect of islet transplantation on the % lymphocytes and lymphocyte subsets in the lymphoid tissues, PBS-treated (Table 20) or W3/25, OX8, OX38 or OX8/OX38 moAb-treated diabetic BB/E rats (Tables 21 - 24 respectively) were compared with the % cells in corresponding islet-transplanted, PBS-treated (Table 25) or W3/25, OX8, OX38 or OX8/OX38 moAb-treated rats (Tables 26 - 29).

The % $CD4^+$ T cells was significantly higher in PLN and the thymus of PBS-treated BB/E rats receiving an intraportal graft compared with rats receiving PBS treatment alone ($p<0.05$).

Table 25. Lymphocyte and lymphocyte subsets (%) in lymphoid tissues of islet-transplanted diabetic BB/E rats receiving PBS-treatment.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)				
Sub-group* (n)	Lymphocyte subset†	PLN	MLN	SPLEEN	PEC	THYMUS
A (8)	lymphocytes	45.9 ± 4.3	35.9 ± 8.3	31.4 ± 6.0	25.1 ± 9.4	87.6 ± 1.8
	$CD5^+$	28.7 ± 4.6	7.4 ± 1.4	4.4 ± 0.8	20.6 ± 8.2	73.9 ± 7.6
	IgM^+	26.4 ± 7.8	49.1 ± 10.1	64.7 ± 5.7	19.7 ± 6.6	5.3 ± 2.6
	$CD8^+$	3.0 ± 0.3	2.7 ± 1.2	5.6 ± 0.9	10.6 ± 5.6	5.3 ± 1.5
	$CD4^+$	22.4 ± 1.4	11.0 ± 1.6	11.0 ± 2.5	47.3 ± 9.5	9.9 ± 0.8
	$CD4^+/CD8^+$	0.3 ± 0.2	1.4 ± 0.6	1.9 ± 1.0	1.0 ± 1.0	55.3 ± 11.9

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

Table 26. The effect of short-term W3/25 moAb treatment on lymphocyte and lymphocyte subsets (%) in lymphoid tissues of islet-transplanted diabetic BB/E rats.

Sub-group* (n)	Lymphocyte subset†	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%) PLN	MLN	SPLEEN	PEC	THYMUS
A (5)	lymphocytes	51.6 ± 9.5	58.4 ± 8.9	50.0 ± 5.5	43.0 ± 6.9	79.2 ± 10.2
	$CD5^+$	21.2 ± 1.3	8.8 ± 0.6	3.8 ± 0.4	3.6 ± 0.6	57.6 ± 17.3
	IgM^+	11.2 ± 5.3	35.4 ± 5.4	56.6 ± 3.3	43.4 ± 10.0	10.6 ± 9.4
	$CD8^+$	4.6 ± 0.5	1.6 ± 0.5	4.6 ± 1.5	1.8 ± 0.4	10.8 ± 2.0
	$CD4^+$	20.8 ± 2.0	13.2 ± 1.7	18.2 ± 1.5	74.8 ± 7.6	14.6 ± 3.7
	$CD4^+/CD8^+$	0.8 ± 0.4	0.6 ± 0.2	2.4 ± 1.0	7.8 ± 4.1	52.0 ± 14.0
B (3)	lymphocytes	58.7 ± 9.4	73.0 ± 2.1	43.0 ± 3.5	49.7 ± 1.8	73.7 ± 9.8
	$CD5^+$	21.3 ± 2.7	7.3 ± 0.7	6.7 ± 1.5	3.3 ± 0.7	73.7 ± 10.7
	IgM^+	27.7 ± 3.4	53.7 ± 10.4	62.3 ± 5.2	70.0 ± 9.5	2.0 ± 0.6
	$CD8^+$	2.3 ± 0.3	0.7 ± 0.3	5.3 ± 0.3	1.0 ± 0.6	12.0 ± 0.0
	$CD4^+$	25.7 ± 2.0	11.7 ± 0.3	20.0 ± 1.0	65.7 ± 22.5	16.7 ± 7.7
	$CD4^+/CD8^+$	0.7 ± 0.3	0.7 ± 0.3	2.7 ± 1.5	3.0 ± 3.0	62.7 ± 11.3

C (1)	lymphocytes	59.0	83.0	44.0	16.0	89.0
	$CD5^+$	16.0	4.0	3.0	36.0	50.0
	IgM^+	7.0	33.0	45.0	12.0	5.0
	$CD8^+$	2.0	1.0	13.0	1.0	26.0
	$CD4^+$	1.0	7.0	7.0	71.0	6.0
	$CD4^+/CD8^+$	1.0	1.0	3.0	3.0	60.0
TOTAL (9)	lymphocytes	54.8 ± 5.8	66.0 ± 5.7	47.0 ± 3.3	42.2 ± 5.0	78.4 ± 6.3
	$CD5^+$	20.7 ± 1.2	7.8 ± 0.6	4.7 ± 0.7	7.1 ± 3.6	62.1 ± 10.1
	IgM^+	16.2 ± 4.1	41.2 ± 5.2	57.2 ± 2.9	48.8 ± 8.7	7.1 ± 5.1
	$CD8^+$	3.6 ± 0.5	1.2 ± 0.3	5.8 ± 1.2	1.4 ± 0.3	12.9 ± 2.0
	$CD4^+$	20.2 ± 2.8	12.0 ± 1.1	17.6 ± 1.6	71.0 ± 8.0	14.3 ± 3.1
	$CD4^+/CD8^+$	0.8 ± 0.2	0.7 ± 0.2	2.3 ± 0.7	5.6 ± 2.4	48.5 ± 9.8

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

Table 27. The effect of short-term OX8 moAb treatment on lymphocyte and lymphocyte subsets (%) in lymphoid tissues of islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)				
Sub-group* (n)	Lymphocyte subset†	PLN	MLN	SPLEEN	PEC	THYMUS
A (1)	lymphocytes	46.0	22.0	31.0	4.0	84.0
	$CD5^+$	28.0	8.0	6.0	12.0	83.0
	IgM^+	47.0	76.0	71.0	49.0	3.0
	$CD8^+$	3.0	2.0	12.0	13.0	10.0
	$CD4^+$	30.0	11.0	18.0	31.0	11.0
	$CD4^+/CD8^+$	0.0	1.0	2.0	1.0	66.0
B (7)	lymphocytes	62.7 ± 6.4	58.6 ± 8.2	50.9 ± 3.8	31.6 ± 8.0	77.9 ± 9.0
	$CD5^+$	27.3 ± 3.8	6.7 ± 1.4	6.0 ± 1.8	8.3 ± 2.7	77.1 ± 8.5
	IgM^+	16.9 ± 4.3	39.9 ± 5.7	54.7 ± 6.6	52.4 ± 9.7	5.6 ± 3.6
	$CD8^+$	3.3 ± 0.4	4.4 ± 2.7	3.4 ± 0.9	3.0 ± 0.6	10.1 ± 1.5
	$CD4^+$	27.1 ± 3.8	10.1 ± 1.9	26.1 ± 7.7	54.7 ± 11.6	15.4 ± 3.4
	$CD4^+/CD8^+$	0.9 ± 0.3	1.0 ± 0.3	4.0 ± 1.6	0.3 ± 0.2	55.3 ± 11.6

C (1)	lymphocytes	50.0	26.0	43.0	53.0	67.0
	$CD5^+$	15.0	5.0	12.0	11.0	-
	IgM^+	18.0	-	-	-	-
	$CD8^+$	21.0	26.0	16.0	13.0	1.0
	$CD4^+$	1.0	2.0	11.0	3.0	15.0
	$CD4^+/CD8^+$	0.0	1.0	4.0	0.0	81.0
TOTAL (9)	lymphocytes	59.4 ± 5.4	50.8 ± 8.1	47.8 ± 3.7	30.9 ± 7.4	77.3 ± 7.0
	$CD5^+$	26.0 ± 3.2	6.9 ± 1.1	6.0 ± 1.4	8.8 ± 2.3	77.9 ± 7.0
	IgM^+	20.3 ± 4.7	40.0 ± 7.4	51.8 ± 7.3	47.4 ± 8.7	5.3 ± 2.9
	$CD8^+$	5.2 ± 2.0	6.6 ± 3.2	5.8 ± 1.7	4.3 ± 1.3	9.1 ± 1.5
	$CD4^+$	24.6 ± 4.1	9.3 ± 1.7	23.6 ± 6.1	46.3 ± 10.7	14.9 ± 2.6
	$CD4^+/CD8^+$	0.7 ± 0.3	1.0 ± 0.2	3.8 ± 1.2	0.3 ± 0.2	59.3 ± 9.4

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

Table 28. The effect of short-term OX38 moAb treatment on lymphocyte and lymphocyte subsets (%) in lymphoid tissues of islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)				
Sub-group* (n)	Lymphocyte subset†	PLN	MLN	SPLEEN	PEC	THYMUS
B (6)	lymphocytes	49.5 ± 6.1	51.5 ± 11.2	46.0 ± 4.5	30.3 ± 9.7	76.3 ± 13.4
	$CD5^+$	25.7 ± 3.1	7.5 ± 1.2	5.3 ± 0.9	5.5 ± 3.1	65.7 ± 9.4
	IgM^+	21.0 ± 4.1	45.3 ± 9.0	60.3 ± 4.1	49.8 ± 10.0	5.3 ± 3.4
	$CD8^+$	3.8 ± 0.8	1.7 ± 0.7	5.8 ± 1.6	2.8 ± 1.1	15.3 ± 3.3
	$CD4^+$	26.3 ± 3.4	18.8 ± 8.1	15.0 ± 1.7	56.3 ± 10.3	7.5 ± 0.8
	$CD4^+/CD8^+$	1.2 ± 0.4	3.5 ± 2.5	2.8 ± 0.7	5.8 ± 5.3	61.3 ± 11.6
C (3)	lymphocytes	43.3 ± 4.4	56.7 ± 15.5	73.3 ± 9.7	9.7 ± 2.0	64.7 ± 11.3
	$CD5^+$	5.7 ± 0.7	24.0 ± 8.0	21.3 ± 15.8	31.0 ± 5.8	51.0 ± 23.1
	IgM^+	58.0 ± 11.5	36.3 ± 10.5	49.3 ± 17.7	18.3 ± 9.0	32.0 ± 28.0
	$CD8^+$	13.7 ± 10.7	15.3 ± 13.3	8.3 ± 5.9	12.7 ± 2.0	15.3 ± 3.3
	$CD4^+$	19.0 ± 8.0	17.0 ± 7.5	12.7 ± 1.8	32.3 ± 11.1	23.7 ± 1.3
	$CD4^+/CD8^+$	5.0 ± 2.1	2.0 ± 0.6	3.0 ± 1.0	21.7 ± 9.8	54.7 ± 6.4

TOTAL (9)	lymphocytes	47.4 ± 4.3	53.2 ± 8.5	55.1 ± 6.1	23.4 ± 7.2	72.4 ± 9.4
	$CD5^+$	19.0 ± 3.9	13.0 ± 3.7	10.7 ± 5.3	14.0 ± 5.0	65.6 ± 9.5
	IgM^+	33.3 ± 7.5	42.3 ± 6.7	56.7 ± 6.0	39.3 ± 8.7	14.2 ± 9.5
	$CD8^+$	8.6 ± 3.5	6.2 ± 4.5	6.7 ± 2.0	6.4 ± 1.8	15.3 ± 2.3
	$CD4^+$	23.9 ± 3.4	18.2 ± 5.6	14.2 ± 1.3	48.3 ± 8.4	12.9 ± 2.8
	$CD4^+/CD8^+$	2.4 ± 0.9	3.0 ± 1.7	2.9 ± 0.5	11.1 ± 5.1	59.1 ± 7.8

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

Table 29. The effect of short-term combined OX8/OX38 moAb treatment on lymphocytes and lymphocyte subsets (%) in lymphoid tissues of islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (%)				
Sub-group* (n)	Lymphocyte subset†	PLN	MLN	SPLEEN	PEC	THYMUS
A (1)	lymphocytes	65.0	52.0	42.0	25.0	90.0
	$CD5^+$	25.0	8.0	4.0	1.0	95.0
	IgM^+	16.0	38.0	72.0	55.0	0.0
	$CD8^+$	4.0	1.0	1.0	16.0	12.0
	$CD4^+$	26.0	10.0	0.0	35.0	8.0
	$CD4^+/CD8^+$	1.0	1.0	1.0	3.0	76.0
B (4)	lymphocytes	38.0 ± 14.7	53.5 ± 17.5	45.0 ± 15.0	15.3 ± 10.3	94.5 ± 2.5
	$CD5^+$	22.3 ± 3.5	13.0 ± 2.0	5.0 ± 1.5	9.0 ± 1.7	86.0 ± 6.0
	IgM^+	13.0 ± 4.6	28.5 ± 6.5	46.3 ± 10.7	41.5 ± 9.5	1.7 ± 0.7
	$CD8^+$	3.3 ± 0.3	4.0 ± 3.5	6.3 ± 2.8	4.7 ± 0.7	11.0 ± 2.6
	$CD4^+$	20.3 ± 4.6	16.0 ± 4.0	20.0 ± 5.0	65.0 ± 15.7	7.7 ± 0.7
	$CD4^+/CD8^+$	0.3 ± 0.3	1.3 ± 1.3	1.7 ± 0.9	4.7 ± 4.7	75.5 ± 2.5

C (4)	lymphocytes	48.0 ± 5.1	68.3 ± 0.6	76.5 ± 5.6	6.3 ± 4.6	73.5 ± 9.7
	$CD5^+$	9.8 ± 5.1	16.5 ± 1.9	7.5 ± 0.3	36.5 ± 9.2	76.8 ± 4.4
	IgM^+	53.8 ± 16.7	37.0 ± 11.3	47.0 ± 8.3	18.5 ± 8.0	3.3 ± 1.1
	$CD8^+$	2.3 ± 0.5	2.0 ± 0.6	3.3 ± 2.3	14.0 ± 2.8	13.0 ± 1.8
	$CD4^+$	20.0 ± 3.5	22.0 ± 3.1	14.0 ± 1.1	43.8 ± 4.3	16.8 ± 4.4
	$CD4^+/CD8^+$	5.0 ± 1.4	2.8 ± 0.8	3.5 ± 1.2	17.5 ± 7.5	64.5 ± 3.4
TOTAL (9)	lymphocytes	47.9 ± 5.2	61.7 ± 4.9	60.4 ± 8.3	12.0 ± 4.7	77.3 ± 7.4
	$CD5^+$	16.4 ± 3.6	14.3 ± 1.7	6.8 ± 0.5	21.6 ± 7.1	72.8 ± 9.8
	IgM^+	32.0 ± 11.4	34.7 ± 6.4	49.9 ± 6.1	30.3 ± 7.5	2.3 ± 0.7
	$CD8^+$	2.9 ± 0.4	3.0 ± 1.4	5.1 ± 1.8	10.8 ± 2.2	12.1 ± 1.3
	$CD4^+$	20.9 ± 2.3	18.6 ± 2.6	14.5 ± 2.9	50.6 ± 7.0	12.3 ± 2.7
	$CD4^+/CD8^+$	2.8 ± 1.1	2.3 ± 0.6	2.5 ± 0.7	10.9 ± 4.6	60.8 ± 8.9

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

† Lymphocytes are expressed as % of total cells. Lymphocyte subsets are expressed as % of lymphocytes.

In group B, the % IgM^+ B lymphocytes was significantly increased in PLN and PEC ($p<0.05$), and the % $CD4^+$ T cells was significantly increased in PLN and MLN of islet-transplanted, W3/25 moAb-treated diabetic BB/E rats ($p<0.05$). Combining the data for groups A, B and C, demonstrated that % IgM^+ B cells was significantly increased in PEC ($p<0.01$), % $CD5^+$ T cells was significantly increased in PLN and MLN ($p<0.001$), % $CD8^+$ T cells was significantly increased in PLN ($p<0.01$) and % $CD4^+$ T cells was significantly increased in the thymus ($p<0.05$) of W3/25 moAb-treated rats receiving a 1° islet graft.

The % $CD5^+$ and $CD4^+$ T cells were significantly increased in PLN and the thymus respectively ($p<0.05$) of islet-transplanted, OX8 moAb-treated BB/E rats in group B. Using the combined data for groups A, B and C, the % $CD5^+$ T cells was significantly greater in PLN and PEC ($p<0.05$) and the % $CD4^+$ T cells was significantly greater in the thymus ($p<0.01$) of OX8 moAb-treated rats receiving an intraportal islet graft.

OX38 moAb-treated diabetic BB/E rats in group B receiving an islet transplant had a significantly greater % $CD5^+$ T cells ($p<0.05$) in PLN compared with OX38 moAb-treated rats. Islet-transplanted diabetic BB/E rats in group C receiving OX38 moAb treatment had a significantly greater % $CD5^+$ T cells in PEC and IgM^+ B cells in PLN ($p<0.05$) compared with OX38 moAb-treated rats and % $CD4^+$ T cells was significantly greater in the thymus ($p<0.001$).

In group B islet-transplanted rats receiving OX8/OX38 moAb treatment, % $CD8^+$ T cells was significantly increased in PLN and % $CD5^+$ T cells was significantly increased in PEC ($p<0.05$) compared with OX8/OX38 moAb-treated rats. The % lymphocytes and $CD5^+$ T cells were significantly increased in the spleen ($p<0.05$) of

islet-transplanted diabetic BB/E rats receiving OX8/OX38 moAb-treatment in Group C, and with respect to the % $CD5^+$ T cells, also in MLN and PEC ($p<0.01$ and $p<0.05$ respectively). The % IgM^+ B cells was significantly increased in PLN of islet-transplanted rats receiving OX8/OX38 moAb-treatment ($p<0.05$) and % $CD8^+$ T cells was significantly increased in MLN and PEC ($p<0.05$) compared with OX8/OX38 moAb-treated rats. The % $CD5^+$ T cells was also significantly increased in MLN ($p<0.01$), PEC and spleen ($p<0.05$) of islet-transplanted, OX8/OX38 moAb-treated rats upon combining the data for groups A, B and C. The % $CD8^+$ T cells was significantly increased in PLN and PEC ($p<0.05$ and $p<0.01$ respectively) and the % $CD4^+$ T cells was significantly increased in MLN and thymus ($p<0.05$) compared with OX8/OX38 moAb-treated rats.

4.3.3.2. Absolute cell numbers

4.3.3.2.1. PBL and PBL subsets in PBS-treated or short-term moAb treated diabetic BB/E rats.

The number of lymphocyte and lymphocyte subsets (expressed as $x10^5$ cells/ml blood) in diabetic BB/E rats receiving PBS treatment or short-term W3/25, OX8, OX38 or OX8/OX38 moAb treatment are shown in Tables 30 - 34 respectively.

$CD8^+$ T cells were significantly reduced 60, 120 ($p<0.05$) and 240 minutes and 24 hours ($p<0.01$) following the priming injection of OX8 moAb and during, at and 1 week after the end of moAb treatment ($p<0.01$) compared with cell numbers treatment. $CD4^+/CD8^+$ T cells were completely depleted during moAb treatment and were significantly reduced 1 week post-moAb treatment ($p<0.05$) compared with cells pre-moAb treatment.

Table 30. PBL and PBL subsets (x10^5 cells/ml blood) in PBS-treated diabetic BB/E rats.

	LYMPHOCYTES and LYMPHOCYTE SUBSETS (x10^5 cells/ml blood)								
Lymphocyte subset	Pre-moAb treatment (day -3)	+60 min	+120 min	+240 min	+24 hr (day -2)	During moAb treatment (day 0)	End of moAb treatment (day 10)	1 week post-moAb	2 weeks post-moAb (death)
lymphocytes	14.79 ± 4.20	11.43 ± 1.35	14.40 ± 0.56	21.10 ± 4.32	18.40 ± 3.14	14.86 ± 2.54	16.85 ± 0.76	9.34 ± 1.34	17.24 ± 4.55
$CD5^+$	0.86 ± 0.30	0.60 ± 0.12	0.63 ± 0.12	1.16 ± 0.34	0.83 ± 0.28	0.74 ± 0.10	1.02 ± 0.04	0.56 ± 0.15	0.40 ± 0.08
IgM^+	7.65 ± 2.54	3.87 ± 0.69	5.19 ± 0.16	6.48 ± 1.34	4.77 ± 2.06	6.60 ± 1.19	5.97 ± 0.87	5.14 ± 0.70	7.73 ± 2.11
$CD8^+$	1.99 ± 0.76	1.15 ± 0.23	1.58 ± 0.33	1.78 ± 0.60	1.91 ± 0.56	2.11 ± 0.50	1.76 ± 0.14	1.00 ± 0.06	4.51 ± 2.52
$CD4^+$	4.43 ± 1.42	4.34 ± 1.40	4.51 ± 0.77	7.53 ± 1.63	4.87 ± 2.15	3.38 ± 0.52	5.60 ± 0.71	3.10 ± 0.55	6.67 ± 0.63
$CD4^+/CD8^+$	1.15 ± 0.28	0.50 ± 0.00	0.68 ± 0.03	0.58 ± 0.06	0.68 ± 0.52	0.60 ± 0.38	1.09 ± 0.10	0.47 ± 0.12	0.83 ± 0.25

Mean ± SEM

Table 31. PBL and PBL subsets (x10^5 cells/ml blood) in short-term W3/25 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES and LYMPHOCYTE SUBSETS (x10^5 cells/ml blood)								
Lymphocyte subset	Pre-moAb treatment (day -3)	+60 min	+120 min	+240 min	+24 hr (day -2)	During moAb treatment (day 0)	End of moAb treatment (day 10)	1 week post-moAb	2 weeks post-moAb (death)
lymphocytes	15.40 ± 3.20	14.30 ± 1.49	19.50 ± 5.48	19.20 ± 3.20	15.59 ± 3.00	13.77 ± 0.89	12.35 ± 1.84	11.46 ± 2.26	17.00 ± 5.10
$CD5^+$	0.66 ± 0.02	0.81 ± 0.29	0.82 ± 0.25	0.64 ± 0.19	0.75 ± 0.24	0.74 ± 0.13	0.69 ± 0.09	0.52 ± 0.01	0.46 ± 0.06
IgM^+	6.63 ± 1.10	5.14 ± 0.96	5.84 ± 1.43	5.23 ± 0.49	5.81 ± 2.06	62.80 ± 0.40	6.72 ± 1.95	4.99 ± 0.86	6.18 ± 0.99
$CD8^+$	1.43 ± 0.46	1.77 ± 0.07	1.94 ± 0.46	1.14 ± 0.36	1.84 ± 0.30	1.73 ± 0.13	1.84 ± 0.50	1.27 ± 0.04	3.63 ± 0.48
$CD4^+$	6.08 ± 1.56	3.99 ± 0.64	6.27 ± 2.05	8.58 ± 2.00	4.22 ± 2.16	3.90 ± 0.84	4.24 ± 0.99	3.75 ± 0.57	4.75 ± 1.03
$CD4^+/CD8^+$	1.48 ± 0.38	0.66 ± 0.06	1.52 ± 0.73	0.94 ± 0.18	1.40 ± 0.98	0.35 ± 0.21	1.43 ± 0.54	0.81 ± 0.44	0.92 ± 0.40

Mean ± SEM

Table 32. PBL and PBL subsets (x10^5 cells/ml blood) in short-term OX8 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES and LYMPHOCYTE SUBSETS (x10^5 cells/ml blood)								
Lymphocyte subset	Pre-moAb treatment (day -3)	+60 min	+120 min	+240 min	+24 hr (day -2)	During moAb treatment (day 0)	End of moAb treatment (day 10)	1 week post-moAb	2 weeks post-moAb (death)
lymphocytes	13.88 ± 1.06	10.86 ± 2.63	10.69 ± 3.56	9.14 ± 3.19	8.83 ± 1.89	9.87 ± 1.48	14.84 ± 1.39	8.55 ± 3.47	10.17 ± 3.82
$CD5^+$	0.75 ± 0.17	0.48 ± 0.01	0.49 ± 0.19	0.37 ± 0.17	0.34 ± 0.18	3.47 ± 3.03	0.90 ± 0.14	0.30 ± 0.15	0.24 ± 0.06
IgM^+	6.47 ± 0.72	2.87 ± 0.98	3.44 ± 1.47	2.64 ± 1.44	2.72 ± 1.83	4.75 ± 0.58	4.90 ± 0.70	4.78 ± 1.68	4.26 ± 1.42
$CD8^+$	1.47 ± 0.26	0.27 ± 0.09[a]	0.32 ± 0.12[a]	0.09 ± 0.05[b]	0.06 ± 0.03[b]	0.01 ± 0.01[b]	0.04 ± 0.04[b]	0.06 ± 0.03[b]	0.62 ± 0.59
$CD4^+$	4.82 ± 1.21	6.26 ± 2.24	3.10 ± 1.20	2.20 ± 1.02	2.80 ± 1.36	3.09 ± 0.22	6.04 ± 1.25	3.67 ± 1.44	3.88 ± 2.02
$CD4^+/CD8^+$	1.02 ± 0.34	0.71 ± 0.22	0.20 ± 0.08	0.11 ± 0.04	0.10 ± 0.10	0[a]	0.12 ± 0.12	0.06 ± 0.03[a]	0.76 ± 0.69

Mean ± SEM

[a] $p<0.05$ and [b] $p<0.01$ compared with pre-moAb treatment.

Table 33. PBL and PBL subsets (x10^5 cells/ml blood) in short-term OX38 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES and LYMPHOCYTE SUBSETS (x10^5 cells/ml blood)								
Lymphocyte subset	Pre-moAb treatment (day -3)	+60 min	+120 min	+240 min	+24 hr (day -2)	During moAb treatment (day 0)	End of moAb treatment (day 10)	1 week post-moAb	2 weeks post-moAb (death)
lymphocytes	11.43 ± 1.34	9.26 ± 0.62	9.60 ± 1.17	9.16 ± 0.30	11.52 ± 2.59	12.97 ± 3.96	10.65 ± 1.98	8.93 ± 3.15	10.72 ± 1.23
$CD5^+$	0.85 ± 0.28	0.57 ± 0.27	0.65 ± 0.29	0.45 ± 0.18	4.54 ± 4.18	0.45 ± 0.15	1.82 ± 1.25	0.57 ± 0.10	0.36 ± 0.12
IgM^+	5.34 ± 0.75	3.06 ± 0.18	3.20 ± 0.15	2.18 ± 0.26	3.12 ± 1.75	5.71 ± 1.70	3.56 ± 0.73	4.35 ± 1.26	4.80 ± 0.62
$CD8^+$	1.33 ± 0.24	0.87 ± 0.20	0.58 ± 0.14	0.25 ± 0.04[a]	0.15 ± 0.08[b]	0.05 ± 0.05[b]	1.27 ± 1.27	0.25 ± 0.13[a]	1.66 ± 0.75
$CD4^+$	3.50 ± 0.77	3.94 ± 0.72	3.40 ± 0.32	3.00 ± 0.86	1.74 ± 0.87	2.92 ± 0.53	4.05 ± 0.89	4.24 ± 1.56	5.19 ± 0.41
$CD4^+/CD8^+$	0.84 ± 0.10	0.47 ± 0.23	0.26 ± 0.14[a]	0.24 ± 0.07[b]	0.05 ± 0.05[b]	0.02 ± 0.02[b]	0.16 ± 0.11[b]	0.32 ± 0.16	1.03 ± 0.28

Mean ± SEM

[a] $p<0.05$ and [b] $p<0.01$ compared with pre-moAb treatment.

Table 34. PBL and PBL subsets ($x10^5$ cells/ml blood) in short-term combined OX8/OX38 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES and LYMPHOCYTE SUBSETS ($x10^5$ cells/ml blood)								
Lymphocyte subset	Pre-moAb treatment (day -3)	+60 min	+120 min	+240 min	+24 hr (day -2)	During moAb treatment (day 0)	End of moAb treatment (day 10)	1 week post-moAb	2 weeks post-moAb (death)
lymphocytes	16.70 ± 2.30	9.65 ± 1.08	10.25 ± 0.81	13.27 ± 1.16	9.74 ± 2.90	13.45 ± 1.19	14.84 ± 0.89	9.93 ± 2.44	15.47 ± 2.58
$CD5^+$	1.19 ± 0.05	0.60 ± 0.30	0.51 ± 0.05[c]	0.60 ± 0.25	0.40 ± 0.12[b]	0.49 ± 0.01[c]	0.72 ± 0.04[b]	0.41 ± 0.16[b]	0.40 ± 0.08[b]
IgM^+	6.13 ± 1.04	3.11 ± 0.68	2.89 ± 0.37	2.78 ± 0.68	3.44 ± 2.18	6.20 ± 0.75	5.08 ± 0.52	4.90 ± 1.30	3.51 ± 1.98
$CD8^+$	1.70 ± 0.30	0.60 ± 0.05[a]	0.37 ± 0.04[a]	0.14 ± 0.07[b]	0.13 ± 0.13[a]	0[b]	0.05 ± 0.05[b]	0.27 ± 0.27[a]	0.28 ± 0.07[b]
$CD4^+$	6.74 ± 3.60	3.70 ± 0.63	3.30 ± 0.76	2.53 ± 0.28	1.32 ± 0.80	2.98 ± 1.31	5.00 ± 0.47	4.38 ± 1.26	5.21 ± 1.30
$CD4^+/CD8^+$	1.68 ± 0.77	0.48 ± 0.34	0.29 ± 0.15	0.04 ± 0.04	0.03 ± 0.03	0	0.22 ± 0.13	0.46 ± 0.33	0.77 ± 0.11

Mean ± SEM

[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ compared with pre-moAb treatment.

$CD8^+$ T cells were significantly reduced 240 minutes ($p<0.05$) and 24 hours ($p<0.01$) following the priming injection of OX38 moAb and remained significantly reduced during ($p<0.01$) and 1 week after the end of moAb treatment ($p<0.05$) compared with cell numbers treatment. $CD4^+/CD8^+$ T cells were significantly reduced 120 ($p<0.05$) and 240 minutes and 24 hours ($p<0.01$) after the priming OX38 moAb injection compared with cells pre-moAb treatment. Cell numbers remained significantly reduced during and at the end of moAb treatment ($p<0.01$).

$CD5^+$ T cells were significantly reduced 120 minutes ($p<0.001$) and 24 hours ($p<0.01$) following the priming injection of combined OX8/OX38 moAb and remained significantly reduced during ($p<0.001$), at and 1 and 2 weeks after the end of moAb treatment ($p<0.01$) compared with $CD5^+$ T cell numbers pretreatment. $CD8^+$ T cells were significantly reduced 60, 120 ($p<0.05$) and 240 minutes ($p<0.01$) and 24 hours ($p<0.05$) following the priming injection of moAb compared with cell numbers pretreatment. During moAb treatment, $CD8^+$ T cells were completely depleted and remained significantly reduced at and 1 week after the end of moAb treatment ($p<0.01$). $CD4^+/CD8^+$ T cells were completely depleted during moAb treatment.

Values for PBS-treated rats (Table 30) were compared to those for W3/25, OX8, OX38 and combined OX8/OX38 moAb-treated rats (Tables 31 - 34 respectively) to determine the absolute effect of each moAb on the number of lymphocytes and lymphocyte subsets.

$CD5^+$ T cells were significantly reduced at the end of moAb treatment in W3/25 moAb-treated rats ($p<0.05$).

$CD8^+$ T cells were significantly reduced 60, 120 and 240 minutes and 24 hours after the priming injection of OX8 moAb ($p<0.05$), during ($p<0.05$) and at the end of moAb treatment and 1 week post-moAb treatment ($p<0.001$) in OX8 moAb-treated rats. $CD4^+/CD8^+$ T cells were significantly reduced 120 and 240 minutes after the priming OX8 moAb injection ($p<0.01$), at the end of moAb treatment ($p<0.01$) and 1 week post-moAb treatment ($p<0.05$) compared with PBS-treated rats.

Lymphocyte numbers were significantly reduced in OX38 moAb-treated rats 120 minutes after the priming injection of moAb and at the end of moAb treatment ($p<0.05$). $CD8^+$ T cells were significantly reduced 120 minutes and 24 hours after the priming injection of OX8 moAb ($p<0.05$), during ($p<0.05$) and 1 week after the end of moAb treatment ($p<0.01$) compared with PBS-treated rats. $CD4^+/CD8^+$ T lymphocyte numbers were significantly reduced 120 minutes and 24 hours after the priming injection of OX38 moAb ($p<0.05$) and at the end of moAb treatment ($p<0.01$).

The combination of OX8/OX38 moAb significantly reduced lymphocyte numbers in diabetic rats 120 minutes after the priming injection of moAb ($p<0.05$) and $CD5^+$ T cells were significantly reduced at the end of moAb treatment ($p<0.01$). $CD8^+$ T cells were significantly reduced 120 minutes and 24 hours following the priming injection of moAb ($p<0.05$), during ($p<0.05$) and at the end of moAb treatment ($p<0.001$) compared with PBS-treated rats. $CD4^+$ T cells were significantly reduced 240 minutes after the priming injection of OX8/OX38 moAb ($p<0.05$). $CD4^+/CD8^+$ T cells were also significantly reduced at this time point and at the end of moAb treatment ($p<0.01$).

4.3.3.2.2. The effect of PBS treatment or short-term moAb treatment on PBL and PBL subsets in islet-transplanted diabetic BB/E rats.

The number of lymphocyte and lymphocyte subsets (expressed as $x10^5$ cells/ml blood) in islet-transplanted diabetic BB/E rats receiving PBS treatment (Table 35) or short-term W3/25, OX8, OX38 or OX8/OX38 moAb treatment are shown in Tables 36 - 39 respectively. Lymphocyte and lymphocyte subset data was analysed after dividing animals into groups A (early failing islet grafts), B (short-term functioning islet grafts) and C (long-term functioning islet grafts) as previously described.

All PBS-treated rats were group A. IgM^+ B cells were significantly increased at failure of the islet graft in PBS-treated rats compared with B lymphocyte numbers at the end of PBS treatment ($p<0.05$).

W3/25 moAb-treated rats in group A and B had significantly increased $CD5^+$ T cell numbers prior to moAb treatment than at death ($p<0.001$ and $p<0.01$ respectively), and in group B at the end of moAb treatment ($p<0.01$). Combining the data for groups A, B and C also demonstrated that $CD5^+$ T cells were significantly higher before moAb treatment than at death ($p<0.001$) and also upon failure of the 1° islet graft ($p<0.05$). Lymphocyte numbers were also significantly greater prior to moAb treatment than at death ($p<0.05$), and upon failure of the 1° islet graft lymphocytes were significantly greater compared with the end of W3/25 moAb treatment ($p<0.05$). At death, $CD8^+$ T cells were significantly greater in W3/25 moAb-treated rats in group A compared with group B ($p<0.001$).

Table 35. PBL and PBL subsets (x 10^5 cells/ml blood) in islet-transplanted diabetic BB/E rats receiving PBS-treatment.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^5 cells/ml blood)					
Sub-group* (n)	Lymphocyte subset	Pre-moAb treatment	Pretransplant of 1° islet graft	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
A (8)	lymphocytes	12.60 ± 1.50	37.19 ± 7.32	17.17 ± 8.66	14.04 ± 2.77	-	11.65 ± 3.36
	$CD5^+$	0.87 ± 0.12	1.53 ± 0.59	0.90 ± 0.20	0.84 ± 0.14	-	0.39 ± 0.12
	IgM^+	5.16 ± 1.08	22.43 ± 6.25	9.60 ± 1.99	4.45 ± 0.87[a]	-	7.78 ± 2.45
	$CD8^+$	0.92 ± 0.15	2.14 ± 0.76	1.32 ± 0.29	0.84 ± 0.28	-	2.15 ± 1.00
	$CD4^+$	4.00 ± 0.82	11.17 ± 3.68	6.96 ± 0.97	4.36 ± 1.42	-	4.22 ± 1.34
	$CD4^+/CD8^+$	0.37 ± 0.12	0.44 ± 0.20	0.77 ± 0.23	0.94 ± 0.58	-	1.29 ± 0.44

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

[a] $p<0.05$ compared with failure of 1° islet graft

Table 36. The effect of short-term W3/25 moAb treatment on PBL and PBL subsets (x 10^5 cells/ml blood) in islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^5 cells/ml blood)					
Sub-group* (n)	Lymphocyte subset	Pre-moAb treatment‡	Pretransplant of 1° islet graft‡	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
A (5)	lymphocytes	22.40 ± 3.70	28.90 ± 11.58	13.60 ± 4.60	22.00 ± 6.00	-	11.30 ± 3.97
	$CD5^+$	0.89 ± 0.11	1.24 ± 0.61	0.58 ± 0.15	0.83 ± 0.27	-	0.26 ± 0.08[a]
	IgM^+	12.83 ± 2.92	17.88 ± 8.2	10.87 ± 4.28	13.11 ± 4.55	-	6.80 ± 2.80
	$CD8^+$	1.70 ± 0.79	2.19 ± 0.75	4.68 ± 0.20	1.81 ± 0.69	-	2.38 ± 0.13
	$CD4^+$	8.12 ± 1.72	10.62 ± 5.22	4.79 ± 1.85	7.80 ± 3.00	-	12.10 ± 8.51
	$CD4^+/CD8^+$	0.36 ± 0.13	0.83 ± 0.38	0.84 ± 0.46	3.16 ± 1.82	-	1.73 ± 0.73
B (3)	lymphocytes	-	-	5.45	13.57 ± 2.77	-	7.36 ± 2.80
	$CD5^+$	-	-	0.16	0.41 ± 0.08[b]	-	0.30 ± 0.12[b]
	IgM^+	-	-	1.63	4.23 ± 1.29	-	3.62 ± 0.54
	$CD8^+$	-	-	0.60	0.76 ± 0.11	-	0.58 ± 0.10[c]
	$CD4^+$			3.70	6.99 ± 3.60	-	3.09 ± 1.71
	$CD4^+/CD8^+$	-	-	0.16	0.65 ± 0.00	-	0.39 ± 0.14

C (1)	lymphocytes	-	-	-	9.57	18.92	8.20
	$CD5^+$	-	-	-	0.38	0.57	0.08
	IgM^+	-	-	-	4.02	7.0	4.35
	$CD8^+$	-	-	-	0.57	1.51	2.62
	$CD4^+$	-	-	-	0.35	9.08	2.38
	$CD4^+/CD8^+$	-	-	-	0.19	0.57	0.25
TOTAL (9)	lymphocytes	-	-	4.60 ± 3.91	18.30 ± 4.10	18.92	9.66 ± 2.34[d]
	$CD5^+$	-	-	0.50 ± 0.15[d]	0.67 ± 0.18	0.57	0.25 ± 0.06[a]
	IgM^+	-	-	9.02 ± 3.80	9.75 ± 3.19	7.00	5.47 ± 1.58
	$CD8^+$	-	-	0.48 ± 0.16	1.39 ± 0.46	1.51	1.81 ± 0.76
	$CD4^+$	-	-	4.57 ± 1.45	7.06 ± 2.00	9.08	8.00 ± 4.79
	$CD4^+/CD8^+$	-	-	0.70 ± 0.38	2.16 ± 1.19	0.57	1.70 ± 0.64

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

‡ The data for pre-moAb treatment and pretransplant of the 1° islet graft is expressed as a total for groups A, B and C.

[d] $p<0.05$, [b] $p<0.01$ and [a] $p<0.001$ compared with pre-moAb treatment.
[c] $p<0.001$ compared with group A.

Table 37. The effect of short-term OX8 moAb treatment on PBL and PBL subsets (x 10^5 cells/ml blood) in islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^5 cells/ml blood)					
Sub-group* (n)	Lymphocyte subset	Pre-moAb treatment‡	Pretransplant of 1° islet graft‡	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
A (1)	lymphocytes	16.74 ± 4.37	18.00 ± 4.64	19.1	9.51	-	6.56
	$CD5^+$	1.04 ± 0.35	1.00 ± 0.30	0.76	0.38	-	0.11
	IgM^+	8.36 ± 2.99	10.70 ± 2.66	9.16	2.95	-	4.58
	$CD8^+$	0.89 ± 0.30	0.03 ± 0.03[a,b]	0.00	0.00	-	0.34
	$CD4^+$	7.20 ± 2.40	5.33 ± 1.54[c]	7.45	7.04	-	2.55
	$CD4^+/CD8^+$	0.30 ± 0.13	0.15 ± 0.07	0.19	0.00	-	0.57
B (7)	lymphocytes	-	-	22.20 ± 3.50	13.43 ± 5.94	-	4.73 ± 1.14[a,d,e]
	$CD5^+$	-	-	1.09 ± 0.19	0.90 ± 0.36	-	0.40 ± 0.28
	IgM^+	-	-	14.87 ± 4.38	6.24 ± 2.42	-	1.90 ± 0.34
	$CD8^+$	-	-	0.27 ± 0.27	0.31 ± 0.28	-	0.44 ± 0.18[d]
	$CD4^+$	-	-	11.40 ± 2.03	4.64 ± 2.27[f]	-	1.63 ± 0.39[a,d,e]
	$CD4^+/CD8^+$	-	-	0.52 ± 0.34	0.28 ± 0.20	-	0.44 ± 0.30

C (1)	lymphocytes	-	-	-	29.14	20.00	18.80
	$CD5^+$	-	-	-	1.46	1.00	0.00
	IgM^+	-	-	-	11.07	7.20	0.00
	$CD8^+$	-	-	-	0.00	0.60	0.00
	$CD4^+$	-	-	-	9.62	10.4	0.00
	$CD4^+/CD8^+$	-	-	-	0.00	1.00	0.00
TOTAL (9)	lymphocytes	-	-	21.59 ± 2.78^g	14.91 ± 4.80	20.00	$6.50 \pm 1.78^{a,d}$
	$CD5^+$	-	-	1.02 ± 0.16	0.90 ± 0.28	1.00	0.32 ± 0.22^f
	IgM^+	-	-	13.73 ± 3.58	6.43 ± 1.94	7.20	1.98 ± 0.46^h
	$CD8^+$	-	-	0.22 ± 0.22	0.23 ± 0.21	0.60	0.39 ± 0.15
	$CD4^+$	-	-	10.63 ± 1.76	5.56 ± 1.79	10.4	1.55 ± 0.37
	$CD4^+/CD8^+$	-	-	0.46 ± 0.30	0.21 ± 0.15	1.00	0.40 ± 0.24

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

‡ The data for pre-moAb treatment and pretransplant of the 1° islet graft is expressed as a total for groups A, B and C.

[a] $p<0.05$ compared with pre-moAb treatment.
[b] $p<0.05$ compared with death (TOTAL).
[c] $p<0.05$ and [c] $p<0.01$ compared with failure of 1° islet graft in group B.
[d] $p<0.05$ compared with pretransplant of 1° islet graft.
[f] $p<0.05$ compared with failure of 1° islet graft.
[g] $p<0.001$ compared with end of moAb treatment.
[h] $p<0.01$ compared with failure of 1° islet graft (TOTAL).

Table 38. The effect of short-term OX38 moAb treatment on PBL and PBL subsets (x 10^5 cells/ml blood) in islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^5 cells/ml blood)					
Sub-group* (n)	Lymphocyte subset	Pre-moAb treatment‡	Pretransplant of 1° islet graft‡	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
B (6)	lymphocytes	19.46 ± 4.10	17.50 ± 3.73	9.94 ± 2.41	13.66 ± 3.10	-	8.39 ± 2.97[a]
	$CD5^+$	0.86 ± 0.18	0.37 ± 0.11[a]	0.54 ± 0.10	0.81 ± 0.25	-	0.27 ± 0.08[a]
	IgM^+	10.80 ± 3.70	10.47 ± 2.43	5.54 ± 2.80	6.34 ± 1.67	-	5.24 ± 2.04
	$CD8^+$	1.10 ± 0.40	0.34 ± 0.24	0.18 ± 0.09[a]	0.15 ± 0.06[a]	-	1.34 ± 0.58
	$CD4^+$	7.58 ± 2.03	4.78 ± 1.42	4.60 ± 0.84	4.90 ± 0.81	-	5.32 ± 2.82
	$CD4^+/CD8^+$	0.72 ± 0.28	0.18 ± 0.10	0.32 ± 0.10	0.30 ± 0.16	-	0.83 ± 0.46
C (3)	lymphocytes	-	-	-	9.23 ± 6.90	34.90 ± 10.19	8.94 ± 4.70
	$CD5^+$	-	-	-	0.52 ± 0.33	1.44 ± 0.56	0.19 ± 0.02[b]
	IgM^+	-	-	-	4.80 ± 3.90	17.10 ± 4.65	3.35 ± 0.60
	$CD8^+$	-	-	-	1.22 ± 1.22	5.69 ± 4.32	0.24 ± 0.02
	$CD4^+$	-	-	-	3.15 ± 2.24	11.81 ± 6.28	1.89 ± 0.32[a]
	$CD4^+/CD8^+$	-	-	-	0.55 ± 0.52	2.37 ± 1.22	0.21 ± 0.04

TOTAL (9)	lymphocytes	-	-	9.94 ± 2.41	12.05 ± 3.04	34.90 ± 10.19	8.70 ± 2.30[a,c]
	$CD5^+$	-	-	0.54 ± 0.10	0.71 ± 0.19	1.44 ± 0.56	0.25 ± 0.06[b,d]
	IgM^+	-	-	5.54 ± 2.80	5.64 ± 1.70	17.10 ± 4.65	4.74 ± 1.54
	$CD8^+$	-	-	0.18 ± 0.09[a]	0.55 ± 0.45	5.69 ± 4.32	1.06 ± 0.46
	$CD4^+$	-	-	4.60 ± 0.84	4.24 ± 0.94	11.81 ± 6.28	4.36 ± 2.17
	$CD4^+/CD8^+$	-	-	0.32 ± 0.10	0.40 ± 0.20	2.37 ± 1.22	0.68 ± 0.50

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

‡ The data for pre-moAb treatment and pretransplant of the 1° islet graft is expressed as a total for groups B and C.

[a] $p<0.05$ and [b] $p<0.01$ compared with pre-moAb treatment.
[c] $p<0.05$ compared with pretransplant of 2° islet graft.
[d] $p<0.05$ compared with failure of 1° islet graft.

Table 39. The effect of short-term combined OX8/OX38 moAb treatment on PBL and PBL subsets (x 10^5 cells/ml blood) in islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^5 cells/ml blood)					
Sub-group* (n)	Lymphocyte subset	Pre-moAb treatment‡	Pretransplant of 1° islet graft‡	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
A (1)	lymphocytes	15.50 ± 2.00	15.72 ± 3.53	14.52	8.65	-	10.50
	$CD5^+$	0.84 ± 0.13	0.57 ± 0.14	1.16	0.78	-	0.21
	IgM^+	5.70 ± 1.23	9.40 ± 3.51	8.13	0.41	-	5.04
	$CD8^+$	1.09 ± 0.27	0.17 ± 0.17[a]	0.15	0.00	-	2.52
	$CD4^+$	5.79 ± 0.74	5.46 ± 1.03	6.10	4.40	-	5.14
	$CD4^+/CD8^+$	0.53 ± 0.13	0.01 ± 0.01[b,c,d]	0.87	0.00	-	1.05
B (4)	lymphocytes	-	-	12.69 ± 2.30	13.94 ± 4.69	-	4.24 ± 1.42[e,f]
	$CD5^+$	-	-	0.94 ± 0.40	0.54 ± 0.04	-	0.16 ± 0.07[e]
	IgM^+	-	-	6.22 ± 0.24	11.10 ± 4.98	-	2.89 ± 1.63
	$CD8^+$	-	-	0	0	-	1.00 ± 0.47
	$CD4^+$	-	-	5.37 ± 0.47	6.11 ± 2.26	-	7.16 ± 5.57
	$CD4^+/CD8^+$	-	-	0	0.20 ± 0.11	-	1.28 ± 1.24

C (4)	lymphocytes	-	-	-	10.70 ± 1.46	24.40 ± 3.95	10.80 ± 2.58
	$CD5^+$	-	-	-	0.59 ± 0.11	0.95 ± 0.30	0.42 ± 0.12
	IgM^+	-	-	-	3.91 ± 0.94	11.41 ± 1.58	7.95 ± 2.35
	$CD8^+$	-	-	-	0.17 ± 0.07[b]	5.22 ± 2.59	0.94 ± 0.13[b]
	$CD4^+$	-	-	-	3.92 ± 0.35[a]	6.48 ± 1.92	5.00 ± 1.19
	$CD4^+/CD8^+$	-	-	-	0.10 ± 0.06[c]	1.90 ± 0.68	0.98 ± 0.61
TOTAL (9)	lymphocytes	-	-	13.30 ± 1.44	11.66 ± 1.82	24.40 ± 3.80	8.63 ± 1.74[a]
	$CD5^+$	-	-	1.01 ± 0.25	0.67 ± 0.09	0.95 ± 0.30	0.30 ± 0.08[b,g]
	IgM^+	-	-	6.86 ± 0.65	6.60 ± 2.14	11.41 ± 1.58	5.22 ± 1.56
	$CD8^+$	-	-	0.05 ± 0.05[b,h]	0.07 ± 0.05[b,h]	5.22 ± 2.59	1.16 ± 0.25
	$CD4^+$	-	-	5.61 ± 0.36	4.80 ± 0.85	6.48 ± 1.92	3.50 ± 0.84[g]
	$CD4^+/CD8^+$	-	-	0.29 ± 0.03	0.12 ± 0.05[a]	1.90 ± 0.68	1.10 ± 0.50

Mean ± SEM

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

‡ The data for pre-moAb treatment and pretransplant of the 1° islet graft is expressed as a total for groups A, B and C.

[a] $p<0.05$, [b] $p<0.01$ and [e] $p<0.001$ compared with pre-moAb treatment.
[c] $p<0.05$ compared with pretransplant of 2° islet graft.
[d] $p<0.05$ and [h] $p<0.001$ compared with death (TOTAL).
[f] $p<0.05$ compared with death. [g] $p<0.05$ compared with failure of 1° islet graft.

$CD8^+$ T cells were significantly greater prior to OX8 moAb treatment compared with pretransplant of the 1° islet graft ($p<0.05$). In group B, lymphocytes were significantly reduced upon killing compared with pre-moAb treatment, pretransplant ($p<0.05$) and failure of the intraportal islet graft ($p<0.01$). $CD8^+$ T cells were significantly higher upon killing of OX8 moAb-treated rats compared with pretransplant of the 1° graft ($p<0.05$). $CD4^+$ T cells were significantly greater at failure of the 1° islet graft compared with pretransplant, end of moAb treatment ($p<0.05$) and upon killing ($p<0.01$). Combining the lymphocyte data for groups A, B and C demonstrated that numbers were significantly reduced at death compared with pre-moAb treatment, pretransplant ($p<0.05$) and failure of the 1° islet graft ($p<0.001$). $CD5^+$ T cells and IgM^+ B cell numbers were both significantly greater at failure of the 1° islet graft compared with cell numbers at death ($p<0.05$ and $p<0.01$ respectively). $CD8^+$ T cells were significantly higher at death than prior to transplantation of the intraportal islet graft ($p<0.05$).

$CD5^+$ T cells were significantly reduced prior to transplantation of the 1° graft in OX38 moAb-treated BB/E rats compared with cell numbers pre-moAb treatment ($p<0.05$). In group B, lymphocyte and $CD5^+$ T cell numbers were significantly greater prior to moAb treatment than at death ($p<0.05$). $CD8^+$ T cells were significantly reduced at the end of moAb treatment and upon failure of the islet graft compared with cell numbers pre-moAb treatment ($p<0.05$). Final $CD5^+$ and $CD4^+$ T cell numbers of OX38 moAb-treated BB/E rats in group C were significantly reduced compared with cell numbers prior to moAb treatment ($p<0.01$ and $p<0.05$ respectively). Combining the lymphocyte and lymphocyte subset values for groups B and C demonstrated that both lymphocytes and $CD5^+$ T cells were significantly reduced at death compared with pre-moAb treatment ($p<0.05$ and $p<0.01$ respectively) and $CD5^+$ T cells were significantly higher at failure of the 1° islet graft

compared with cell numbers upon killing ($p<0.05$). Lymphocyte numbers were significantly higher at the time of transplantation of the 2° islet graft under the kidney capsule compared with the time of death ($p<0.05$).

$CD8^+$ and $CD4^+/CD8^+$ T cell numbers were significantly lower in OX8/OX38 moAb-treated rats prior to transplantion of the 1° graft than pretransplant ($p<0.05$ and $p<0.01$ respectively). In group A, both lymphocyte subsets were completely depleted at the end of moAb treatment. In group B, $CD8^+$ T cells were also depleted at the end of moAb treatment and remained depleted at failure of the islet graft. $CD4^+/CD8^+$ T cells were also depleted at failure of the intraportal islet graft. Both lymphocyte and $CD5^+$ T cells were significantly reduced prior to islet transplantation and at death compared with cell numbers pre-moAb treatment ($p<0.001$ and $p<0.05$ respectively). Lymphocyte numbers were significantly greater at islet graft failure compared with the final values at death ($p<0.05$). CD5+ T cells were significantly reduced at the end of moAb treatment ($p<0.01$). In group C, $CD8^+$ T cell numbers were significantly lower at the end of OX8/OX38 moAb-treatment and upon killing compared with pre-moAb treatment ($p<0.01$) and pretransplantation of the 1° islet graft ($p<0.01$) respectively. $CD4^+$ T cells were significantly lower at the end of OX8/OX38 moAb treatment compared with pre-moAb treatment ($p<0.05$). $CD4^+/CD8^+$ T cells were significantly higher at pre-moAb treatment compared with the end of moAb treatment ($p<0.05$) and at the end of treatment compared with pretransplantion of the 2° islet graft under the kidney capsule ($p<0.05$). The combined data for groups A, B and C demonstrated that lymphocytes and $CD5^+$ T cell numbers were both significantly higher pre-moAb treatment than at the time of death ($p<0.05$ and $p<0.01$ respectively). $CD5^+$ T cells were also significantly higher at the time of 1° graft failure compared with cell numbers upon killing of OX8/OX38 moAb-treated rats ($p<0.05$). $CD8^+$ T cell numbers were significantly lower at the

time of 1° graft failure and at the end of moAb treatment compared with cell numbers pre-moAb treatment ($p<0.01$). Upon killing, $CD8^+$ T cells were significantly higher at the time of 1° graft failure and at the end of moAb treatment ($p<0.001$). $CD4^+$ T cells were significantly higher at the time of 1° graft failure compared with cell numbers at death ($p<0.05$). $CD4^+/CD8^+$ T cells were significantly lower at the end of treatment compared with pre-moAb treatment ($p<0.05$) but were significantly higher at death compared with pretransplantion of the 1° islet graft ($p<0.05$).

The lymphocyte and lymphocyte subset numbers of PBS-treated BB/E rats receiving a 1° islet graft (Table 35) were compared with islet-transplanted animals receiving short-term W3/25, OX8, OX38 or OX8/OX38 moAb treatment (Tables 36 - 39).

OX8 moAb-treated diabetic BB/E rats had significantly decreased numbers of lymphocytes and $CD8^+$ T cells prior to transplantation of a 1° islet graft compared with PBS-treated rats ($p<0.05$). In group B and the combined group of A, B and C, $CD8^+$ T cells were significantly lower at the time of 1° graft failure ($p<0.05$).

Prior to transplantation of the 1° islet graft, lymphocytes and $CD8^+$ T cells were significantly lower in OX38 moAb-treated BB/E rats ($p<0.05$). In group B, $CD8^+$ T cell numbers were significantly lower in OX38 moAb-treated rats at the time of islet graft failure and at the end of moAb treatment compared with PBS-treated rats ($p<0.01$ and $p<0.05$ respectively). Combining the data for groups B and C demonstrated that $CD8^+$ T cells were significantly reduced in moAb-treated rats at the time of 1° graft failure ($p<0.01$).

Lymphocyte and $CD8^+$ T cell numbers in OX8/OX38 moAb-treated diabetic rats were significantly reduced prior to transplantation of the 1° graft compared with cell

numbers pre-moAb treatment ($p<0.05$). In group B, $CD8^+$ T cells were significantly lower at islet graft failure and at the end of moAb treatment ($p<0.001$ and $p<0.05$ respectively). $CD4^+/CD8^+$ T cells were significantly lower at the time of islet graft failure in OX8/OX38 moAb-treated rats ($p<0.05$). In group C and the combined group of A, B and C, $CD8^+$ T cell numbers were significantly lower in moAb-treated rats at the end of treatment compared with PBS-treated rats ($p<0.05$) and also at the time of 1° graft failure in the combined group ($p<0.01$).

There was no significant difference between the numbers of lymphocytes and lymphocyte subsets in islet-transplanted diabetic BB/E rats receiving PBS treatment (Table 35) or short-term W3/25, OX8, OX38 or OX8/OX38 moAb treatment (Tables 36 - 39 respectively) when compared with diabetic BB/E rats receiving PBS (Table 30) or the corresponding moAb alone (Tables 31 - 34).

4.3.3.2.3. Lymphocytes and lymphocyte subsets in lymphoid tissues of PBS-treated or short-term moAb-treated diabetic BB/E rats.

The number of lymphocyte and lymphocyte subsets (generally expressed as $x10^6$ cells/g tissue) in lymphoid tissues of diabetic BB/E rats receiving PBS treatment (Table 40) or short-term W3/25, OX8, OX38 or OX8/OX38 moAb treatment are shown in Tables 41 - 44 respectively. Values for PBS-treated rats were compared to those for W3/25, OX8, OX38 or OX8/OX38 moAb-treated rats to determine the effect of each moAb had on the numbers of lymphocytes and lymphocyte subsets in the lymphoid tissue.

Lymphocyte and $CD4^+$ T cell numbers in the spleen of W3/25 moAb-treated rats were significantly reduced compared with PBS-treated control rats ($p<0.05$).

Table 40. Lymphocytes and lymphocyte subsets (x10^6 cells/g tissue) in lymphoid tissues of PBS-treated (control) diabetic BB/E rats.

	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^6 cells/g tissue) ‡				
Lymphocyte subset	PLN	MLN	Spleen	PEC	Thymus
Lymphocytes	109.42 ± 18.58	220.86 ± 94.67	82.48 ± 10.99	21.95 ± 5.33	1095.41 ± 83.65
$CD5^+$	15.32 ± 1.74	8.70 ± 2.48	4.83 ± 1.04	0.46 ± 0.32	845.26 ± 25.85
IgM^+	22.04 ± 9.01	119.66 ± 82.09	42.83 ± 4.77	5.10 ± 2.63	10.95 ± 0.84
$CD8^+$	2.94 ± 0.70	2.39 ± 0.79	8.40 ± 2.23	10.80 ± 0.28	202.96 ± 83.44
$CD4^+$	18.87 ± 1.99	18.43 ± 5.41	12.26 ± 1.19	12.30 ± 3.63	60.66 ± 10.05
$CD4^+/CD8^+$	0.80 ± 0.80	1.67 ± 0.85	3.42 ± 2.06	0	680.27 ± 124.51

Mean ± SEM. n = 3.

‡ Values for PEC are expressed as 10^5/ml volume.

Table 41. Lymphocytes and lymphocyte subsets (x10^6 cells/g tissue) in lymphoid tissues of short-term W3/25 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^6 cells/g tissue) ‡				
Lymphocyte subset	PLN	MLN	Spleen	PEC	Thymus
Lymphocytes	101.25 ± 6.37	223.15 ± 54.21	40.18 ± 8.47	13.34 ± 3.13	1296.21 ± 207.61
$CD5^+$	11.02 ± 0.77	9.33 ± 1.79	1.67 ± 0.88	0.09 ± 0.05	909.18 ± 22.64
IgM^+	12.10 ± 2.98	101.52 ± 0.32	15.78 ± 3.70	2.63 ± 1.56	55.26 ± 22.96
$CD8^+$	2.03 ± 0.13	2.23 ± 0.01	2.89 ± 0.87	0.50 ± 0.18	121.82 ± 36.53
$CD4^+$	15.59 ± 1.09	20.86 ± 0.06	5.48 ± 1.83	4.00 ± 1.41	70.92 ± 12.71
$CD4^+/CD8^+$	1.60 ± 0.53	2.63 ± 0.00	1.65 ± 1.18	0.05 ± 0.05	1037.43 ± 144.50

Mean ± SEM. n = 3.

‡ Values for PEC are expressed as 10^5/ml volume.

Table 42. Lymphocytes and lymphocyte subsets (x10^6 cells/g tissue) in lymphoid tissues of short-term OX8 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^6 cells/g tissue) ‡				
Lymphocyte subset	PLN	MLN	Spleen	PEC	Thymus
Lymphocytes	100.53 ± 45.2	173.62 ± 0.93	47.45 ± 8.21	10.35 ± 1.07	885.28 ± 155.78
$CD5^+$	8.27 ± 1.11	7.41 ± 0.01	1.47 ± 0.86	0.26 ± 0.10	680.25 ± 118.68
IgM^+	38.36 ± 29.48	77.17 ± 0.54	27.27 ± 5.04	2.84 ± 1.82	18.38 ± 7.81
$CD8^+$	2.11 ± 1.22	0.31 ± 0.31	1.52 ± 0.92	0.15 ± 0.10	77.68 ± 15.88
$CD4^+$	14.00 ± 2.56	15.61 ± 0.06	5.73 ± 0.82	6.73 ± 2.69	42.71 ± 13.66
$CD4^+/CD8^+$	1.96 ± 1.21	1.61 ± 0.01	2.14 ± 1.02	0	730.80 ± 120.09

Mean ± SEM. n = 3.

‡ Values for PEC are expressed as 10^5/ml volume.

Table 43. Lymphocytes and lymphocyte subsets (x10^6 cells/g tissue) in lymphoid tissues of short-term OX38 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^6 cells/g tissue) ‡				
Lymphocyte subset	PLN	MLN	Spleen	PEC	Thymus
Lymphocytes	135.25 ± 33.97	206.97 ± 52.73	58.48 ± 5.56	3.27 ± 0.91	1174.25 ± 162.99
$CD5^+$	20.30 ± 5.75	14.67 ± 6.48	3.24 ± 0.96	0.79 ± 0.57	967.49 ± 107.44
IgM^+	12.90 ± 4.47	71.90 ± 28.59	30.57 ± 4.26	2.67 ± 1.43	11.74 ± 1.63
$CD8^+$	3.23 ± 1.00	1.45 ± 0.89	3.84 ± 0.68	0.39 ± 0.15	95.72 ± 5.30
$CD4^+$	22.94 ± 4.92	21.12 ± 4.96	12.52 ± 4.47	4.66 ± 1.96	76.32 ± 5.77
$CD4^+/CD8^+$	1.35 ± 0.34	2.07 ± 0.53	2.70 ± 1.12	0.32 ± 0.16	952.55 ± 122.97

Mean ± SEM. n = 3.

‡ Values for PEC are expressed as 10^5/ml volume.

Table 44. Lymphocytes and lymphocyte subsets (x10^6 cells/g tissue) in lymphoid tissues of short-term combined OX8/OX38 moAb-treated diabetic BB/E rats.

	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^6 cells/g tissue) ‡				
Lymphocyte subset	PLN	MLN	Spleen	PEC	Thymus
Lymphocytes	183.80 ± 85.85	228.35 ± 44.68	40.00 ± 5.00	13.26 ± 4.21	1060.13 ± 234.05
$CD5^+$	22.03 ± 8.54	14.14 ± 2.50	1.70 ± 1.00	0.49 ± 0.30	842.09 ± 153.36
IgM^+	19.98 ± 12.93	76.25 ± 20.83	23.67 ± 1.97	4.99 ± 0.73	18.79 ± 7.26
$CD8^+$	3.67 ± 1.72	2.28 ± 0.45	2.23 ± 0.48	0.38 ± 0.14	86.00 ± 6.44
$CD4^+$	24.95 ± 8.98	21.21 ± 3.18	7.02 ± 1.07	5.74 ± 1.10	53.32 ± 10.07
$CD4^+/CD8^+$	2.17 ± 1.51	2.28 ± 0.45	1.95 ± 0.15	1.45 ± 0.90	889.46 ± 221.17

Mean ± SEM. n = 3.

‡ Values for PEC are expressed as 10^5/ml volume.

OX8 moAb-treated BB/E rats had significantly reduced $CD5^+$ T cells in PLN ($p<0.05$) and $CD8^+$ T cell numbers were significantly reduced in the spleen and PEC ($p<0.05$).

Lymphocyte and $CD4^+$ T cell numbers were significantly reduced in the spleen of OX8/OX38 moAb-treated rats compared with PBS-treated control rats ($p<0.05$).

4.3.3.2.4. The effect of PBS treatment or short-term moAb treatment on lymphocytes and lymphocyte subsets in lymphoid tissues of islet-transplanted diabetic BB/E rats.

The number of lymphocyte and lymphocyte subsets (generally expressed as $x10^6$ cells/g tissue) in the lymphoid tissues of islet-transplanted diabetic BB/E rats receiving PBS treatment (Table 45) or short-term W3/25, OX8, OX38 or OX8/OX38 moAb treatment are shown in Tables 46 - 49 respectively. MoAb treatments had no significant effect on the numbers of lymphocytes and lymphocyte subset in the lymphoid tissues when compared with PBS-treated rats.

Numbers of lymphocytes and lymphocyte subsets in the lymphoid tissues of islet-transplanted diabetic BB/E rats receiving PBS treatment (Table 45) or short-term W3/25, OX8, OX38 or OX8/OX38 moAb treatment (Tables 46 - 49 respectively) were significantly lower than the number of lymphocytes in the lymphoid tissue of non-islet-transplanted BB/E rats receiving corresponding PBS (Table 40) or short-term W3/25, OX8, OX38 or OX8/OX38 moAb treatment (Tables 41 - 44 respectively) in most cases.

Table 45. Lymphocyte and lymphocyte subsets (x 10^6 cells/g tissue) in lymphoid tissues of islet-transplanted diabetic BB/E rats receiving PBS-treatment.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^6 cells/g tissue)‡				
Sub-group* (n)	Lymphocyte subset	PLN	MLN	SPLEEN	PEC	THYMUS
A (8)	lymphocytes	29.14 ± 4.94	53.97 ± 9.56	17.34 ± 8.43	0.97 ± 0.22	221.17 ± 67.98
	$CD5^+$	6.88 ± 1.67	4.67 ± 0.96	1.01 ± 0.58	0.12 ± 0.05	186.26 ± 62.52
	IgM^+	7.03 ± 1.99	25.82 ± 7.61	7.80 ± 3.90	0.25 ± 0.12	4.52 ± 0.91
	$CD8^+$	0.85 ± 0.15	0.67 ± 0.05	0.84 ± 0.59	0.08 ± 0.05	16.31 ± 6.84
	$CD4^+$	6.81 ± 1.45	5.59 ± 0.83	3.09 ± 1.14	0.54 ± 0.18	16.18 ± 5.67
	$CD4^+/CD8^+$	0.10 ± 0.05	0.45 ± 0.17	0.37 ± 0.35	0.02 ± 0.02	145.72 ± 62.80

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

‡ Values for PEC are expressed as 10^5/ml volume.

Table 46. The effect of short-term W3/25 moAb treatment on lymphocyte and lymphocyte subsets (x 10^6 cells/g tissue) in lymphoid tissues of islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^6 cells/g tissue)‡				
Sub-group* (n)	Lymphocyte subset	PLN	MLN	SPLEEN	PEC	THYMUS
A (5)	lymphocytes	30.32 ± 6.25	59.72 ± 8.96	23.25 ± 5.99	11.60 ± 9.40	351.53 ± 93.79
	$CD5^+$	6.34 ± 1.42	5.35 ± 1.06	0.91 ± 0.23	0.42 ± 0.27	220.56 ± 90.44
	IgM^+	4.41 ± 2.64	19.88 ± 2.95	13.35 ± 3.86	6.81 ± 5.68	8.32 ± 3.39
	$CD8^+$	1.34 ± 0.25	0.99 ± 0.30	0.98 ± 0.52	0.14 ± 0.09	44.00 ± 12.71
	$CD4^+$	7.69 ± 1.56	7.89 ± 1.48	4.45 ± 1.20	8.30 ± 6.60	44.91 ± 14.98
	$CD4^+/CD8^+$	0.20 ± 0.12	0.50 ± 0.22	0.65 ± 0.27	2.30 ± 2.24	223.46 ± 72.74
B (3)	lymphocytes	16.18 ± 6.18	47.19 ± 6.87	51.73 ± 18.17	4.14 ± 2.90	54.36 ± 27.00
	$CD5^+$	4.14 ± 2.54	3.78 ± 0.55	3.82 ± 2.48	0.15 ± 0.13	45.49 ± 30.17
	IgM^+	5.08 ± 2.08	30.07 ± 3.46	34.44 ± 9.61	3.12 ± 2.10	0.82 ± 0.00
	$CD8^+$	0.37 ± 0.07	0.47 ± 0.07	2.59 ± 0.91	0.08 ± 0.06	6.52 ± 3.24
	$CD4^+$	4.34 ± 2.14	5.67 ± 0.83	9.82 ± 2.77	3.39 ± 3.17	5.19 ± 2.73
	$CD4^+/CD8^+$	0.16 ± 0.06	0.47 ± 0.07	2.25 ± 1.24	0	40.23 ± 19.98

C (1)	lymphocytes	105.02	517.92	41.36	17.60	706.66
	$CD5^+$	16.8	20.71	1.24	6.34	353.33
	IgM^+	7.35	170.91	18.61	2.10	35.33
	$CD8^+$	2.10	5.18	5.38	0.18	187.73
	$CD4^+$	1.05	36.25	2.90	12.50	42.4
	$CD4^+/CD8^+$	1.05	5.18	1.24	0.53	424.00
TOTAL (9)	lymphocytes	36.14 ± 10.85	113.83 ± 57.98	32.63 ± 6.85	10.50 ± 5.86	321.63 ± 91.90
	$CD5^+$	6.91 ± 1.75	6.88 ± 2.09	1.68 ± 0.68	1.06 ± 0.77	193.39 ± 65.25
	IgM^+	4.94 ± 1.67	41.30 ± 18.68	19.28 ± 4.47	5.30 ± 3.50	9.82 ± 4.34
	$CD8^+$	1.19 ± 0.25	1.38 ± 0.58	1.93 ± 0.66	0.13 ± 0.06	52.10 ± 21.16
	$CD4^+$	6.02 ± 1.35	10.88 ± 3.75	5.60 ± 1.29	7.60 ± 4.15	34.56 ± 11.06
	$CD4^+/CD8^+$	0.29 ± 0.13	1.08 ± 0.60	1.12 ± 0.38	1.51 ± 1.40	202.72 ± 61.31

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

‡ Values for PEC are expressed as 10^5/ml volume.

Table 47. The effect of short-term OX8 moAb treatment on lymphocyte and lymphocyte subsets (x 10^6 cells/g tissue) in lymphoid tissues of islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^6 cells/g tissue)‡				
Sub-group* (n)	Lymphocyte subset	PLN	MLN	SPLEEN	PEC	THYMUS
A (1)	lymphocytes	40.35	11.33	16.11	0.52	97.61
	$CD5^+$	11.30	0.91	0.97	0.06	81.01
	IgM^+	18.97	8.61	11.44	0.26	2.93
	$CD8^+$	1.21	0.23	1.93	0.07	9.76
	$CD4^+$	12.11	1.25	2.90	0.16	10.74
	$CD4^+/CD8^+$	0.00	0.11	0.32	0.01	64.42
B (7)	lymphocytes	36.01 ± 7.66	33.12 ± 11.50	23.42 ± 4.96	3.74 ± 2.39	245.84 ± 70.43
	$CD5^+$	10.42 ± 3.52	2.76 ± 1.07	1.42 ± 0.36	0.44 ± 0.38	217.54 ± 70.59
	IgM^+	4.38 ± 1.65	10.19 ± 3.34	14.42 ± 4.45	1.46 ± 0.60	4.79 ± 1.56
	$CD8^+$	0.99 ± 0.23	1.00 ± 0.60	0.84 ± 0.20	0.07 ± 0.02	32.97 ± 10.66
	$CD4^+$	10.17 ± 3.27	3.39 ± 1.46	4.57 ± 0.71	2.30 ± 1.55	25.79 ± 6.40
	$CD4^+/CD8^+$	0.34 ± 0.20	0.32 ± 0.19	0.75 ± 0.33	0.85 ± 0.85	174.48 ± 60.53

C (1)	lymphocytes	15.00	0.12	30.53	2.65	101.07
	$CD5^+$	2.25	0.08	18.93	2.12	0.00
	IgM^+	2.70	0.01	3.66	0.29	92.06
	$CD8^+$	3.15	0.03	4.88	2.44	1.01
	$CD4^+$	0.15	0.00	3.36	0.08	15.18
	$CD4^+/CD8^+$	0.00	0.00	1.22	0.00	81.95
TOTAL (9)	lymphocytes	34.16 ± 6.34	27.03 ± 9.70	23.40 ± 3.97	3.26 ± 1.86	213.30 ± 57.94
	$CD5^+$	9.61 ± 2.84	2.25 ± 0.88	3.31 ± 1.97	0.59 ± 0.35	178.20 ± 60.25
	IgM^+	5.82 ± 2.08	9.99 ± 2.74	12.90 ± 3.60	1.19 ± 0.49	14.28 ± 9.80
	$CD8^+$	1.25 ± 0.29	0.81 ± 0.48	1.41 ± 0.48	0.07 ± 0.02	26.84 ± 9.13
	$CD4^+$	9.27 ± 2.75	2.78 ± 1.19	4.25 ± 0.59	1.82 ± 1.23	22.94 ± 5.25
	$CD4^+/CD8^+$	0.26 ± 0.16	0.26 ± 0.15	0.75 ± 0.27	0.66 ± 0.66	151.94 ± 48.6

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

‡ Values for PEC are expressed as 10^5/ml volume.

Table 48. The effect of short-term OX38 moAb treatment on lymphocyte and lymphocyte subsets (x 10^6 cells/g tissue) in lymphoid tissues of islet-transplanted diabetic BB/E rats.

Sub-group* (n)	Lymphocyte subset	LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^6 cells/g tissue)‡ PLN	MLN	SPLEEN	PEC	THYMUS
B (6)	lymphocytes	18.50 ± 4.46	57.60 ± 18.95	29.39 ± 7.25	4.83 ± 2.47	302.36 ± 98.94
	$CD5^+$	4.65 ± 1.35	5.12 ± 1.80	1.39 ± 0.33	0.14 ± 0.08	237.85 ± 76.75
	IgM^+	3.25 ± 0.81	19.15 ± 5.63	18.68 ± 5.12	2.89 ± 1.54	4.78 ± 1.67
	$CD8^+$	0.71 ± 0.16	0.62 ± 0.41	1.51 ± 0.54	0.16 ± 0.09	39.59 ± 13.97
	$CD4^+$	4.83 ± 1.45	6.69 ± 2.34	3.92 ± 0.85	2.41 ± 1.23	24.39 ± 9.24
	$CD4^+/CD8^+$	0.22 ± 0.05	0.93 ± 0.26	0.97 ± 0.42	0.81 ± 0.75	219.22 ± 72.96
C (3)	lymphocytes	17.88 ± 6.81	75.36 ± 42.95	88.30 ± 28.64	3.80 ± 1.52	224.06 ± 142.36
	$CD5^+$	0.93 ± 0.31	12.16 ± 6.78	9.93 ± 3.53	1.35 ± 0.70	160.93 ± 101.77
	IgM^+	14.5 ± 6.26	33.78 ± 18.26	53.58 ± 24.88	0.62 ± 0.43	13.13 ± 8.25
	$CD8^+$	1.14 ± 0.44	1.45 ± 0.78	3.26 ± 1.58	0.54 ± 0.28	43.30 ± 32.98
	$CD4^+$	4.41 ± 2.32	18.49 ± 10.83	12.05 ± 4.88	1.31 ± 0.88	22.63 ± 10.70
	$CD4^+/CD8^+$	1.17 ± 0.66	2.03 ± 1.34	2.08 ± 0.27	0.60 ± 0.35	109.32 ± 59.95

TOTAL (9)	lymphocytes	18.30 ± 3.49	61.65 ± 18.32	49.02 ± 13.66	4.50 ± 1.66	276.26 ± 77.06
	$CD5^+$	3.41 ± 1.07	7.47 ± 2.56	4.14 ± 1.79	0.55 ± 0.29	173.53 ± 59.71
	IgM^+	6.91 ± 2.67	24.02 ± 6.85	30.31 ± 9.82	2.13 ± 1.07	7.57 ± 3.04
	$CD8^+$	0.85 ± 0.18	0.93 ± 0.31	2.10 ± 0.64	0.29 ± 0.12	40.73 ± 13.2
	$CD4^+$	4.69 ± 1.15	10.62 ± 3.99	6.63 ± 2.03	2.00 ± 0.86	23.80 ± 6.72
	$CD4^+/CD8^+$	0.54 ± 0.25	1.29 ± 0.46	1.34 ± 0.34	0.74 ± 0.50	182.59 ± 53.41

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

‡ Values for PEC are expressed as 10^5/ml volume.

Table 49. The effect of short-term combined OX8/OX38 moAb treatment on lymphocyte and lymphocyte subsets (x 10^6 cells/g tissue) in lymphoid tissues of islet-transplanted diabetic BB/E rats.

		LYMPHOCYTES AND LYMPHOCYTE SUBSETS (x 10^6 cells/g tissue)‡				
Sub-group* (n)	Lymphocyte subset	PLN	MLN	SPLEEN	PEC	THYMUS
A (1)	lymphocytes	24.25	0.73	8.61	4.50	145.98
	$CD5^+$	6.06	0.06	0.34	0.05	136.68
	IgM^+	3.88	0.28	6.20	2.48	0.00
	$CD8^+$	0.97	0.01	0.09	0.72	17.52
	$CD4^+$	6.30	0.07	0.00	1.58	11.68
	$CD4^+/CD8^+$	0.24	0.01	0.09	0.14	110.94
B (4)	lymphocytes	17.83 ± 12.88	91.66 ± 61.97	11.44 ± 10.48	2.40 ± 1.49	378.39 ± 155.30
	$CD5^+$	4.70 ± 2.23	10.40 ± 8.03	0.78 ± 0.75	0.15 ± 0.06	308.00 ± 159.20
	IgM^+	1.10 ± 0.57	21.19 ± 15.67	6.49 ± 6.15	1.52 ± 0.70	4.30 ± 1.06
	$CD8^+$	0.54 ± 0.38	1.71 ± 0.03	0.47 ± 0.42	0.10 ± 0.06	48.50 ± 21.95
	$CD4^+$	3.82 ± 2.70	11.63 ± 8.48	3.33 ± 3.19	1.63 ± 1.06	30.45 ± 13.92
	$CD4^+/CD8^+$	0.01 ± 0.01	0.21 ± 0.21	0.01 ± 0.01	0.25 ± 0.25	267.65 ± 138.22

C (4)	lymphocytes	49.85 ± 21.39	135.67 ± 32.42	101.17 ± 27.77	4.60 ± 3.03	207.36 ± 44.98
	$CD5^+$	8.10 ± 6.77	21.32 ± 4.50	7.49 ± 2.07	1.60 ± 1.19	119.72 ± 44.13
	IgM^+	16.16 ± 3.53	41.21 ± 14.66	54.40 ± 18.29	0.80 ± 0.44	23.81 ± 18.12
	$CD8^+$	1.27 ± 0.72	2.61 ± 0.87	1.49 ± 0.23	0.84 ± 0.67	28.18 ± 9.35
	$CD4^+$	7.81 ± 1.27	27.11 ± 3.99	15.43 ± 4.97	2.29 ± 1.20	29.11 ± 2.84
	$CD4^+/CD8^+$	1.73 ± 0.26	3.96 ± 1.58	2.65 ± 0.58	0.70 ± 0.38	136.76 ± 33.76
TOTAL (9)	lymphocytes	34.64 ± 12.22	99.57 ± 28.62	56.35 ± 21.50	3.80 ± 1.54	263.82 ± 64.79
	$CD5^+$	6.44 ± 3.30	15.16 ± 4.36	4.08 ± 1.62	0.87 ± 0.62	192.45 ± 65.43
	IgM^+	9.28 ± 3.09	29.64 ± 10.48	30.41 ± 12.57	1.25 ± 0.40	13.51 ± 9.27
	$CD8^+$	0.96 ± 0.38	1.98 ± 0.59	0.93 ± 0.28	0.55 ± 0.34	34.47 ± 9.43
	$CD4^+$	6.13 ± 1.27	18.82 ± 5.03	8.97 ± 3.54	1.95 ± 0.87	27.43 ± 5.25
	$CD4^+/CD8^+$	0.90 ± 0.34	2.35 ± 1.13	1.34 ± 0.56	0.46 ± 0.22	182.62 ± 54.04

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

‡ Values for PEC are expressed as 10^5/ml volume.

4.3.4. Stimulation indices.

The stimulation indices of PBS-treated or short-term W3/25, OX8, OX38 or OX8/OX38 moAb-treated diabetic BB/E rats are shown in Table 50. The indices prior to moAb treatment were not significantly different during treatment. However, at the end of moAb treatment all the stimulation indices were lower compared with values prior to and during moAb treatment although this reduction was only significant with OX38 and OX8/OX38 moAb-treatment ($p<0.05$). One week post-moAb treatment, all stimulation indices had increased from the end of treatment and this reached significance ($p<0.05$) in OX8/OX38 moAb-treated rats. Upon killing two weeks post-moAb treatment, the stimulation indices were not significantly different from the pre-moAb treatment values. The stimulation indices for PBS-treated and moAb-treated diabetic BB/E rats were not significantly different at any time point.

The stimulation indices of islet-transplanted diabetic BB/E rats receiving PBS treatment or short-term W3/25, OX8, OX38 or OX8/OX38 moAb treatment are shown in Table 51.

The stimulation indices of PBS-treated control rats were significantly lower upon failure of the islet graft, end of PBS treatment and death compared with pretreatment ($p<0.01$) and pretransplant ($p<0.05$) of the intraportal islet graft.

The stimulation index of W3/25 moAb-treated BB/E rats significantly decreased prior to intraportal transplantation of the 1° islet graft compared with pre-moAb treatment ($p<0.01$) and remained significantly decreased upon failure of the islet graft, at the end of moAb treatment and death ($p<0.01$) in group A. Stimulation indices at the

Table 50. The effect of short-term moAb treatment on stimulation indices of diabetic BB/E rats (non-islet-transplanted).

Moab treatment	STIMULATION INDEX				
	Pre-moAb treatment (day -3)	During moAb treatment (day 0) †	End of moAb treatment (day 10)	1 week post-moAb treatment	2 weeks post-moAb treatment
None (PBS)	10.7 ± 4.8	23.0 ± 8.2	4.5 ± 2.4	15.9 ± 7.4	6.7 ± 3.0
W3/25	9.1 ± 5.5	28.5 ± 12.1	4.3 ± 2.4	10.5 ± 4.5	8.3 ± 5.7
OX8	12.1 ± 4.7	13.0 ± 8.7	10.5 ± 8.5	13.1 ± 5.7	12.2 ± 8.1
OX38	12.1 ± 4.0	19.1 ± 4.3 *	3.8 ± 1.2	14.8 ± 5.9	8.3 ± 2.2
OX8/OX38 (combined)	12.7 ± 8.2	22.4 ± 8.4 *	2.0 ± 0.6	15.1 ± 4.4 *	13.4 ± 7.8

Mean ± SEM. n = 3 for each group.

* $p<0.05$ compared with end of moAb treatment.

† corresponding to transplantation of a 1° graft in islet-transplanted diabetic BB/E rats.

Table 51. The effect of short-term moAb treatment and islet transplantion on stimulation indices of diabetic BB/E rats.

MoAb treatment (n)	Sub-group* (n)	STIMULATION INDEX					
		Pre-moAb treatment‡	Pretransplant of 1° islet graft‡	Failure of 1° islet graft	End of moAb treatment	Pretransplant of 2° islet graft	Death
None (8)	A (8)			3.1 ± 0.8[b,d]	4.0 ± 1.2[b,d]	-	4.4 ± 1.8[b,d]
(PBS)	B (0)	30.2 ± 7.2	16.3 ± 4.4	-	-	-	-
	C (0)			-	-	-	-
W3/25 (9)	A (5)			3.2 ± 1.6[b]	4.1 ± 0.8[b]	-	4.2 ± 1.7[b]
	B (3)	32.6 ± 7.3	7.5 ± 2.2[b]	3.4	6.0 ± 4.0[a]	-	7.0 ± 0.4[b]
	C (1)			-	0.9	8.6	7.0
OX8 (9)	A (1)			5.1	9.4	-	3.4
	B (7)	25.0 ± 7.4	10.8 ± 3.3	13.5 ± 2.6[g]	5.1 ± 1.0[a]	-	7.7 ± 3.2
	C (1)			-	0.9	4.2	12.5
OX38 (9)	A (0)			-	-	-	-
	B (6)	28.1 ± 8.6	10.0 ± 3.1	8.4 ± 3.4	6.9 ± 2.1[a]	-	4.3 ± 0.8[a]
	C (3)			-	6.3 ± 0.8[a]	7.5	6.7 ± 2.6[a]

OX8/OX38 (9)	A (1)			4.7	13.3	-	5.9
	B (4)	13.8 ± 5.5	10.7 ± 2.6	1.1 ± 0.3[a,e]	5.5 ± 2.0	-	3.8 ± 2.0
	C (4)			-	6.8 ± 3.0	16.8 ± 3.1	11.2 ± 3.7
TOTAL (44)	A (15)			3.4 ± 0.6[c,f,g]	7.0 ± 1.3[c,d]	-	4.4 ± 1.0[c,f]
	B (20)	25.9 ± 3.3	11.0 ± 1.4[c]	8.5 ± 2.0[c]	5.9 ± 0.9[c,e]	-	5.8 ± 1.3[c,e]
	C (9)			-	5.3 ± 1.8[c,d]	9.7 ± 1.5[c]	9.4 ± 1.9[c,h]

* Diabetic BB/E rats were divided into 3 groups determined by the period of normoglycaemia following an intraportal islet graft. Group A : early graft failures (< 10 days). Group B : short-term functioning grafts (≥ 10 days). Group C : long-term functioning grafts receiving a 2° islet graft under the kidney capsule.

‡ The stimulation indices for pre-moAb treatment and pretransplant of the 1° islet graft are expressed as a total for groups A, B and C.

[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ compared with pre-moAb treatment.
[d] $p<0.05$, [e] $p<0.01$ and [f] $p<0.001$ compared with pretransplant of 1° islet graft.
[g] $p<0.05$ compared with end of moAb treatment.
[h] $p<0.05$ compared with group A.

end of moAb treatment and death in group B were also significantly lower than the pre-moAb treatment value ($p<0.05$ and $p<0.01$ respectively). The single W3/25 moAb-treated BB/E rat in group C had a lower stimulation index at the end of moAb treatment compared with groups B and C.

OX8 moAb-treated BB/E rats had a lower stimulation index prior to transplantation of the 1° islet graft compared with pre-moAb treatment although this did not reach significance. The stimulation index was significantly lower at the end of moAb treatment compared with pre-moAb treatment for rats in group B ($p<0.05$). The stimulation index at the end of moAb treatment was highest for the diabetic BB/E rat in group A and lowest for the single rat in group C. The stimulation index at the time of failure of the 2° islet graft in this latter group was 19 which was higher than the value observed in group B upon failure of the 1° islet graft.

The stimulation index of OX38 moAb-treated diabetic BB/E rats decreased from pre-moAb treatment to pretransplant of the 1° islet although this did not reach significance until the end of moAb treatment ($p<0.05$). The stimulation index remained significantly decreased after this time point until death ($p<0.05$). The stimulation index at failure of the 1° islet graft in group B was not significantly different from the values at pre-moAb treatment and pretransplant of the intraportal islet graft.

After treatment with a combination of OX8/OX38 moAb, diabetic BB/E rats demonstrated a lower stimulation index than pre-moAb treatment although this decrease was not significant. The stimulation index upon failure of the 1° islet graft in group B animals was significantly lower than at pre-moAb treatment and pretransplant

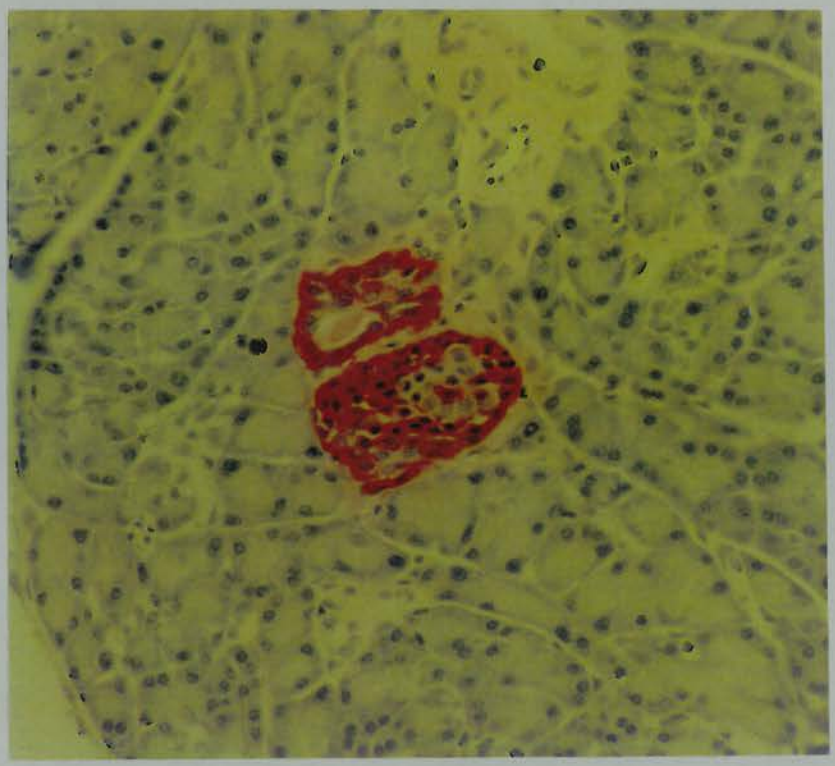
of the graft. The diabetic BB/E rat in group A had a higher stimulation index at the end of moAb treatment than animals in group B and C.

Upon combining the data for groups A, B and C, the stimulation index prior to transplantation of the 1° islet graft was found to be significantly lower compared with the value pre-moAb treatment ($p<0.001$) and remained significantly lower thereafter in groups A, B and C ($p<0.001$). The stimulation index was also significantly lower at the time of 1° islet graft failure in group A ($p<0.001$), the end of moAb treatment in groups A ($p<0.05$), B ($p<0.01$) and C ($p<0.05$) and at the time of death in groups A ($p<0.001$) and B ($p<0.05$) compared with pretransplantation of the 1° islet graft. At the time of death, the stimulation index of group C was significantly higher ($p<0.05$) than the value for group A indicating that the stimulation index eventually increased with time in recipients maintaining long-term functioning islet grafts although this value was not significantly different from the stimulation index prior to transplantation of the 1° islet graft.

4.3.5. Histology

Representative immunohistochemical staining of pancreas, liver and kidney of diabetic BB/E rats receiving a 1° intraportal islet graft (and a 2° islet graft under the kidney capsule in animals maintaining long-term functioning 1° grafts) are shown in Figure 11(a)-(h). Figure 11(a) shows the pancreas of an established (>140 days) diabetic BB/E rat. Islets are small and widely separated and only a mild chronic inflammatory cell infiltrate is noted in relation to them. Immunoperoxidase stains for glucagon are strongly positive and negative for insulin. Light microscopy of the liver of a BB/E rat upon failure of a short-term functioning intraportal islet graft is shown in Figure 11(b). There are several foci of moderate to severe fatty changes associated

(a)



(b)

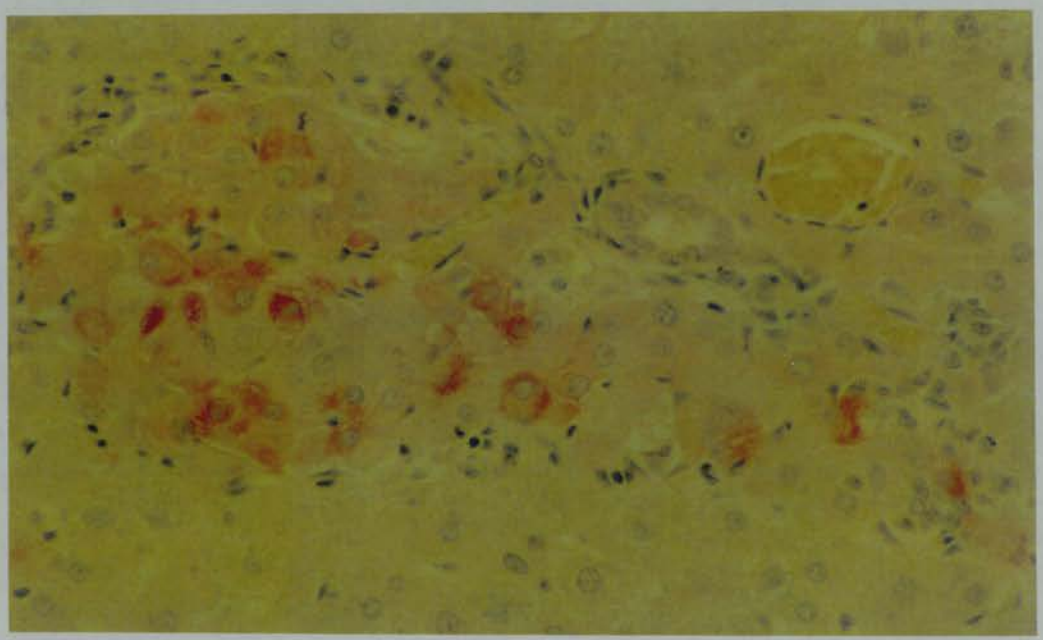
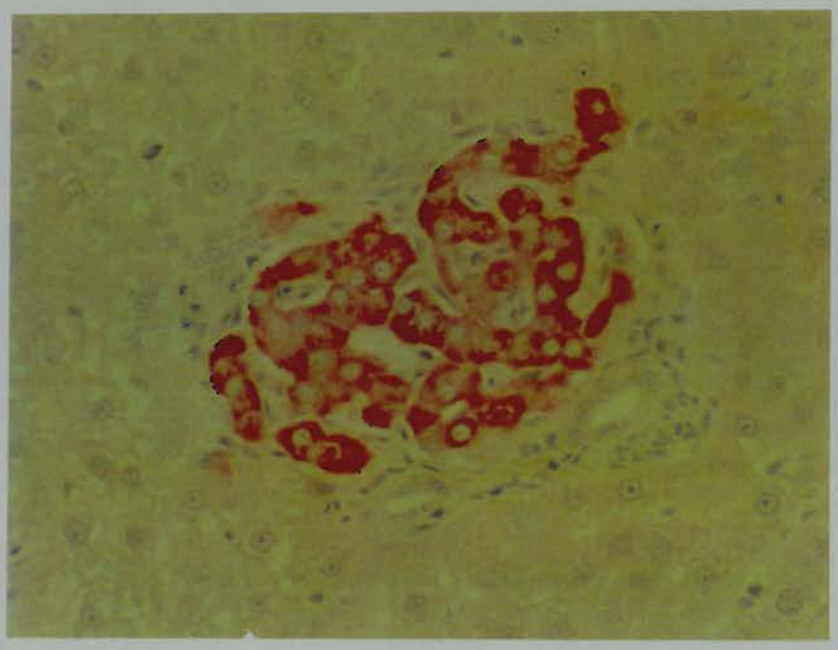


Figure 11(a) Islet cells from pancreas of an established (>140 days) diabetic BB/E rat stained for glucagon. No positivity for insulin was observed. x 400. (b) Islet cells from a short-term functioning intraportal graft 34 days after transplantation into an established diabetic BB/E rat receiving OX38 moAb treatment. Insulin positivity was observed in approximately 50% of islet cells which were surrounded by chronic inflammatory cells comprising mainly of lymphocytes and macrophages. No glucagon positivity was visible. x 400.

(c)



(d)

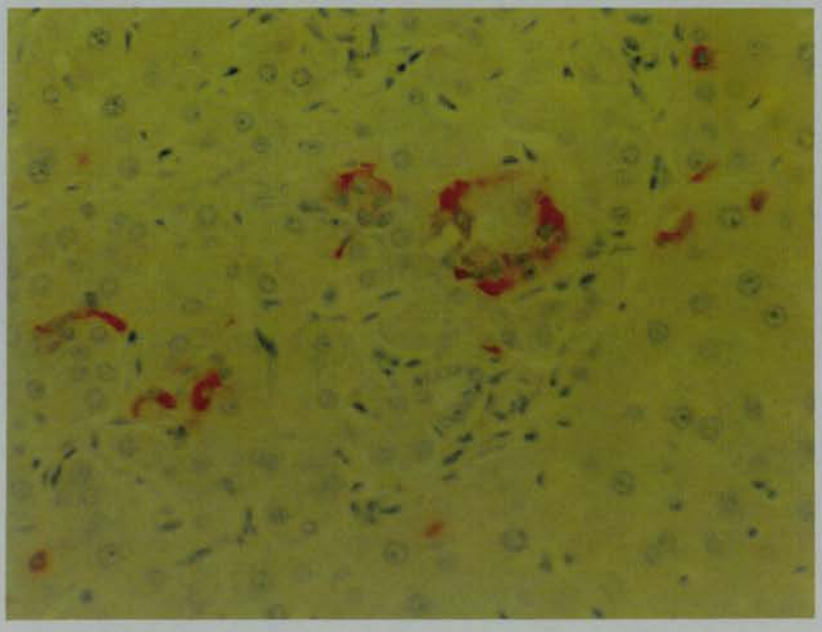
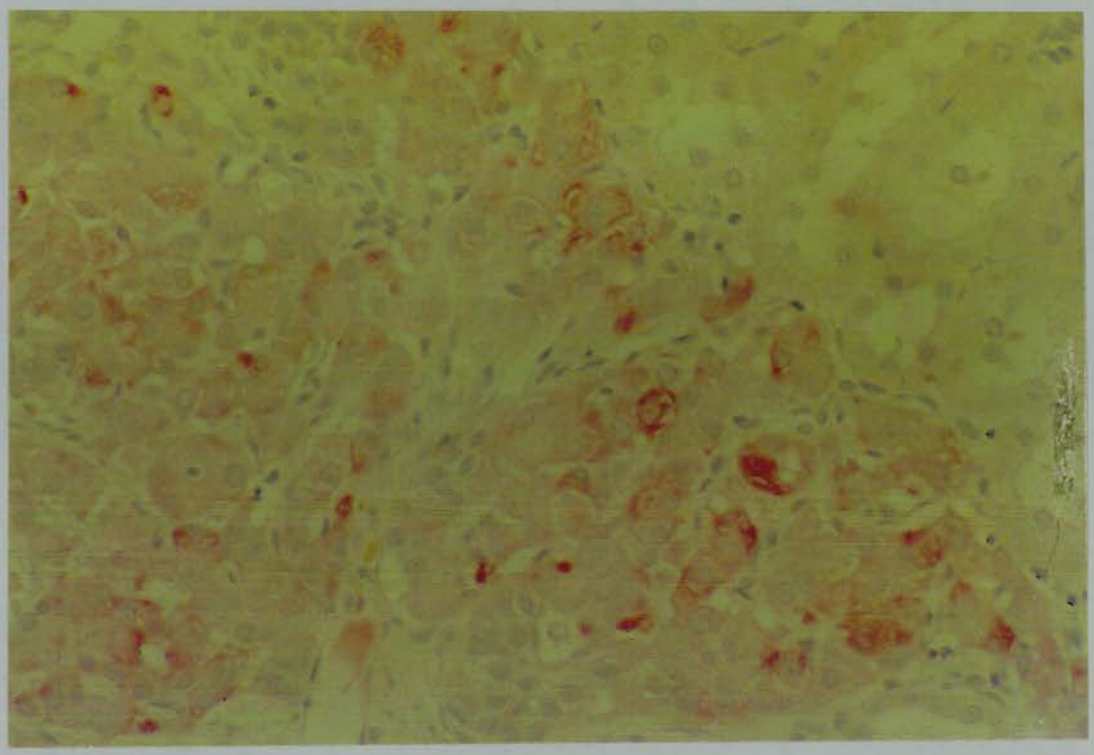


Figure 11. Islet cells from a long-term functioning intraportal graft 98 days after transplantation into an established diabetic BB/E rat receiving combined OX8/OX38 moAb treatment. Insulin positivity was visible (c) and occasional glucagon positivity was observed (d). x 400.

(e)



(f)

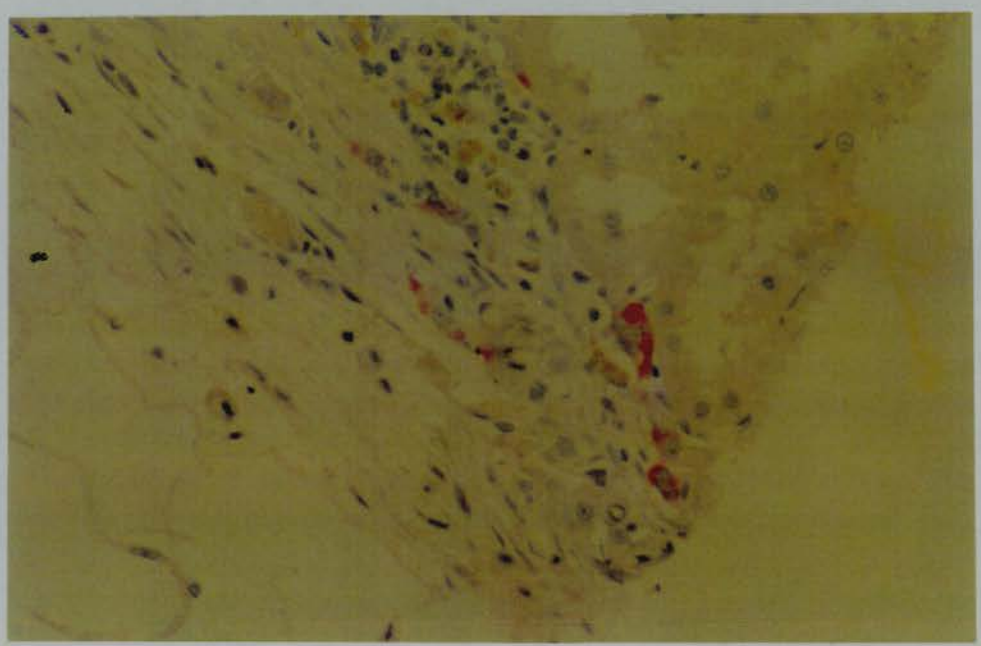
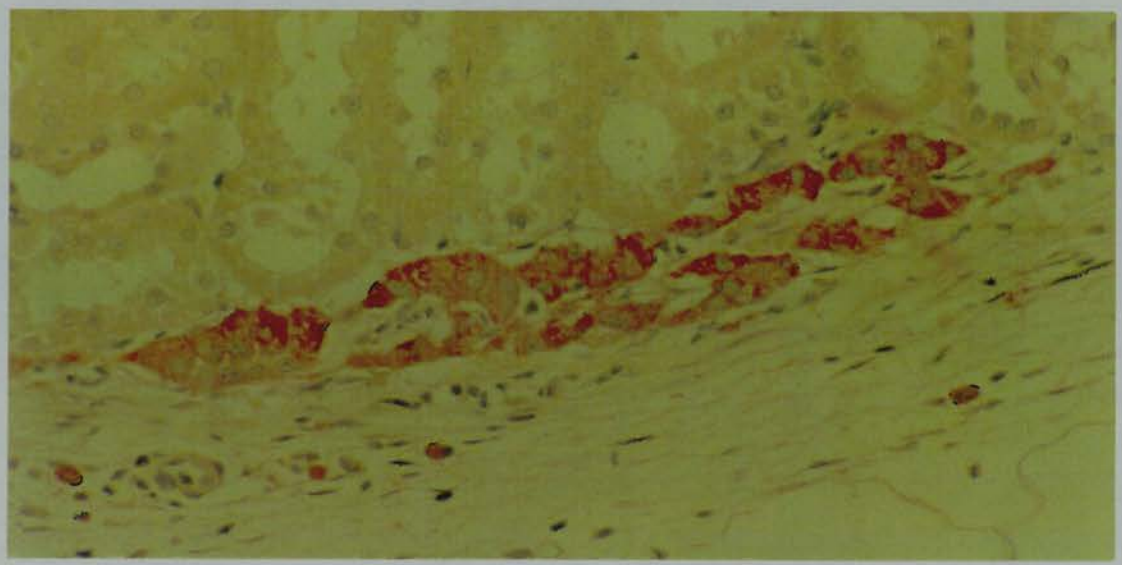


Figure 11. Islet cells from a graft 14 days after transplantation under the kidney capsule of a diabetic BB/E rat receiving no moAb treatment (control). Immunoperoxidase staining for insulin showed faint positivity for insulin (e). Focal positivity for glucagon was occasionally observed (f). A moderate chronic inflammatory cell infiltrate was noted in and around islet cells. x 400.

(g)



(h)

Figure 11. Islet cells from a 2° graft under the kidney capsule 34 days after transplantation into a diabetic BB/E rat maintaining a long-term functioning 1° intraportal islet graft (125 days) following combined OX8/OX38 moAb treatment. Insulin positivity (g) and focal glucagon positivity (h) was observed and there was a chronic inflammatory cell infiltrate adjacent to islet cells in the perinephric fat. x 400.

with small numbers of islets. Immunoperoxidase stains for insulin are mildly positive for insulin and negative for glucagon. A moderate chronic inflammatory cell infiltrate is observed around the islets. Figure 11(c) shows the liver of a BB/E rat maintaining a long-term functioning intraportal islet graft. The overall architecture of the liver was normal and only mild fatty changes were present associated with several clusters of islets in the portal triads. Only a sparse chronic inflammatory cell infiltrate was present in relation to the islets and islet cells were strongly positive for insulin and only occasional positivity for glucagon is observed (d). Diabetic BB/E rats receiving a single islet graft under the kidney capsule acted as controls to those moAb-treated rats receiving a 2° islet graft under the kidney capsule. In the former group, diabetic rats were normoglycaemic for a mean of 3 ± 2 days and the immunohistochemistry of a representative islet-bearing kidney is shown in Figure 11(e) and (f). A thick layer of islet cells were seen beneath the kidney capsule and around the capsule was a moderate chronic inflammatory cell infiltrate. Immunoperoxidase stains for insulin are only occasionally positive (e) and stains for glucagon are generally negative although glucagon-positive cell are occasionally observed at the periphery of the islet cell (f). The islet-bearing kidney of a diabetic BB/E rat maintaining a long-term functioning islet graft is shown in Figure 11(g) and (h). There was a thin layer of islets under the kidney capsule and a chronic inflammatory cell infiltrate including foreign body giant cells, macrophages and basophils was present in the adjacent perinephric fat . Immunoperoxidase stains for insulin were strongly positive (g) and stains for glucagon showed focal positivity only (h).

4.4 DISCUSSION

Transplantation of pancreatic islets for the treatment of diabetes mellitus in the insulin-dependent autoimmune BB rat may fail as a result of two distinct immune

responses : allograft rejection resulting from histocompatibility differences between donor and recipient and recurrence of the autoimmune disease responsible for the initial destruction of the recipients native pancreatic β-cells. Naji et al (869) initially described recurrent autoimmune insulitis in islet-transplanted BB rats that were made tolerant to donor-specific tissue. This phenomenon was subsequently observed by Sibley and colleagues (14) who reported recurrence of diabetes in human recipients of segmental pancreas isografts transplanted between identical twins discordant for IDDM.

IDDM in the spontaneously diabetic BB/E rat has a T lymphocyte-dependent autoimmune aetiology similar to that in human IDDM. Using moAb directed against specific T lymphocyte subsets directly involved in β-cell destruction may prevent the destruction of islets following transplantation into the diabetic BB rat. In this study, the ability of a short-course of non-depleting (W3/25) or depleting (OX38) anti-CD4 moAb or depleting anti-CD8 (OX8) moAb either alone or combined with OX38 moAb to prevent recurrence of IDDM following intraportal transplantation of an islet graft was investigated.

The liver was chosen as the site for implantation of the 1° islet graft rather than under the kidney capsule, which is a proposed immunologically privileged site in the BB rat (653), since islets transplanted intraportally become revascularised as early as 4 days after implantation (870). In contrast, islets implanted under the kidney capsule only become revascularised 4 weeks after implantation in the rat (871). Early revascularisation is essential to ensure an adequate supply of oxygen and nutrients to transplanted islets and transport of secreted insulin to the bloodstream of the recipient. Secretion of insulin into the portal vein is also more physiological than secretion into arterial blood by islets transplanted under the kidney capsule.

On the day of intraportal islet transplantation a half dose of insulin was administered to recipient rats to avoid implantation of islets into a hyperglycaemic environment and immediate stress of islet β-cells. The detrimental effect of hyperglycaemia on transplanted islets has been reported (872). After transplantation (day 0), no further exogenous insulin was given. Blood glucose levels were normalised in islet-transplanted diabetic BB/E rats one day following transplantation.

This study demonstrated that a short course (14 days) of treatment with depleting moAb directed against the CD8 cell surface marker, OX8 or the CD4 cell surface marker OX38, either singly or in combination, significantly prolonged the survival of MHC-incompatible Wistar or WH islets intraportally transplanted into established diabetic BB/E rats. Although outbred Wistar and WH rats are MHC-incompatible with BB/E rats it should be noted that these animals share the $RT1^{u}$ haplotype (i.e. MHC class I homology). The non-depleting moAb W3/25 directed against the CD4 cell surface marker did not significantly prolong islet graft survival when compared with PBS-treated BB/E rats. Despite the overall poor survival of islets transplanted to W3/25 moAb-treated rats, a single BB/E rat maintained long-term normoglycaemia following an intraportal islet graft and subsequently received a 2° islet graft under the kidney capsule. This animal was still normoglycaemic (blood glucose concentration = 4.1 mmol/l) when killed 34 days later. Markmann et al (682) reported a mean graft survival of 25 ± 16 days following intraportal islet transplantation in W3/25 moAb-treated BB rats made tolerant to donor tissue which compared well with the mean survival time observed in this study (21 ± 13 days) despite a different treatment regimen. Markmann injected islet-transplanted rats three-times weekly with W3/25 moAb throughout the period of study (100 days) and the concentration of moAb was not determined. MoAb treatments were also compared after separating islet-transplanted diabetic BB/E rats into three groups (A, B and C) determined by

the periods of normoglycaemia following intraportal islet grafting. The χ^2 test for rectangular contingency tables confirmed that islet grafts survived significantly longer after transplantation to OX8, OX38 and OX8/OX38 moAb-treated diabetic BB/E rats compared with islet-transplanted control rats. Islets transplanted into OX38 moAb-treated rats were also shown to survive significantly longer than islets transplanted into W3/25 moAb-treated rats as evidenced by long-term normoglycaemia. This observation demonstrates that although W3/25 moAb competes with OX38 moAb for binding to the same determinant(s) on rat CD4 cell surface marker (665), treatment with a depleting rather than a non-depleting anti-CD4 moAb significantly improved graft survival. 33% of OX38 moAb-treated rats maintained long-term functioning 1° islet grafts and subsequently received 2° islet grafts under the kidney capsule. All animals remained normoglycaemic. Seydel et al (679) reported permanent survival (>115 days) of islet allografts intraportally transplanted into STZ-diabetic rats following a brief (4 days) course of OX38 moAb treatment. The priming dose (5 mg/kg) was lower than the dose used in this study (10 mg). Seydel also assessed the effect of OX8 moAb on islet survival after intraportal transplantation to STZ-diabetic rats. Using the same treatment protocol, anti-CD8 moAb treatment was shown to prolong the survival of islet allografts only short-term and all treated animals rejected their grafts within 30 days of transplantation (mean = 25 ± 4 days). The mean islet graft survival observed using OX8 moAb in this study was comparable (27 ± 9 days) and 89% of treated animals remained normoglycaemic past the end of OX8 moAb treatment. However, of these, 88% failed within 2 weeks of treatment ending. Only a single OX8 moAb-treated BB/E rat maintained a long-term functioning intraportal islet graft and was borderline hyperglycaemic (blood glucose concentration = 12.2 mmol/l) prior to transplantation of a 2° islet graft under the kidney capsule. Blood glucose concentration was initially normalised but a hyperglycaemic state was observed 9 days after implantation of the

2° islet graft, suggesting 500 islets transplanted to this site were insufficient to achieve long-term normoglycaemia. In contrast, Markmann and colleagues (682) reported that all OX8 moAb-treated BB rats remained normoglycaemic for >100 days following intraportal transplantation of approximately 1500 WF islets. Neonatal tolerance to donor-specific tissues was induced prior to islet transplantation in these animals and BB rats were treated three-times weekly with OX8 moAb until termination at 100 days post-transplantation. In this study, the majority of islet-transplanted rats only returned to a hyperglycaemic state after completion of OX8 moAb treatment suggesting that continued treatment may have further increased survival of islet grafts similar to the results of Markmann et al (682).

Treatment of islet-transplanted BB/E rats with a combination of OX8 and OX38 moAb was the most successful treatment and 44% of rats maintained long-term functioning grafts as evidenced by persistent normoglycaemia. However, this result was not significantly different from either of the moAb treatments alone. In contrast, Seydel et al (679) reported that the combination of OX8 and OX38 moAb abrogated the protective effect of anti-CD4-mediated islet allograft survival in the rat and suggested this finding demonstrated the need of a regulator $CD8^+$ cell in the survival of intraportally transplanted islets in diabetic rats receiving OX38 moAb treatment.

In each group of OX8, OX38 or combined OX8/OX38 moAb-treated BB/E rats, one animal was killed whilst still normoglycaemic due to megacolon, an incidence of 7% in the 44 rats studied. This incidence was approximately 5-fold greater than the incidence of megacolon in the diabetic BB/E rat colony recently reported (873). BB/E rats were in a similar age range in both studies although the larger numbers studied by Meehan and colleagues may give a better representation of megacolon incidence in this colony. Since idiopathic megacolon in the BB rat involves an

inflammatory process including T lymphocytes and macrophages leading to both muscle and nerve destruction in the bowel wall, it would seem likely that anti-CD4 and anti-CD8 moAb treatment would decrease the incidence of megacolon rather than exacerbate the condition.

To determine the effect of moAb treatment on lymphocytes and lymphocyte subset numbers, FACS analysis was performed on PBL and lymphoid tissues. The non-depleting nature of W3/25 moAb observed in this study is in agreement with several previous reports (239,683,684). However, despite the absence of $CD4^+$ cell depletion, 4 animals maintained functioning intraportal islet grafts beyond the end of treatment and 1 animal which received a 2° graft under the kidney capsule was still normoglycaemic at death (122 days after 1° islet transplantation). Interestingly, although $CD4^+$ cell numbers were not significantly decreased in PBL, this single animal (group C) had the lowest $CD4^+$ cell numbers in all lymphoid tissues which may have contributed to the prolonged islet survival observed. It should be noted that despite the observation that W3/25 does not significantly deplete $CD4^+$ cells, this moAb was effective in reversing the disease course of another animal model of autoimmune disease, experimental allergic encephalomyelitis (683,684). Administration of W3/25 moAb also prolonged heart allograft survival across combined MHC and non-MHC barriers in the rat in the absence of marked clearance of $CD4^+$ cells in PBL (680). This was in contrast to the findings of Claesson et al (681) who failed to prolong the survival of anastomotic heart grafts in a semiallogeneic system with a single injection of W3/25 moAb. This discrepancy may be due to strain differences in the rats used, the degree of antigenic disparity or variation in dose and potency of moAb. These findings, including those of the present study suggest that the (limited) inhibitory effect of W3/25 moAb on graft survival may be attributed to interference with activation, either by blockage of T cell receptor

binding to its target cells or by blocking an essential CD4-mediated activation signal, without clearing. Alternatively, it has been suggested by Bank et al (874) that anti-CD4 antibodies themselves transmit a negative signal and depletion of $CD4^+$ cells may not be an essential requirement for induced unresponsiveness.

Administration of depleting OX8 moAb caused an immediate (within 60 minutes) and often complete depletion of $CD8^+$ cells in PBL and was accompanied by a significant reduction in $CD4^+/CD8^+$ cells. The small population of double-stained cells are thought to be immature thymic precursors which mature into $CD4^+$ or $CD8^+$ T cells. The contribution of these cells to islet graft survival (if any) in BB rats is unknown but may serve to lower the pool of $CD4^+$ and $CD8^+$ T cell precursors in PBL. At death there was no depletion of $CD8^+$ or $CD4^+/CD8^+$ T cells in any of the lymphoid tissues and this observation may be time-related since two weeks after the end of OX8 moAb treatment, $CD8^+$ and $CD4^+/CD8^+$ lymphocytes were not significantly lower in PBL than the values prior to moAb treatment indicating repopulation of both cell types. Interestingly, it was in this period of time that the majority (78%) of intraportal islet grafts failed in OX8 moAb-treated BB/E rats. This study implicates the involvement of a $CD8^+$-bearing cell in islet graft destruction in this animal model. OX8 moAb treatment caused sufficient reduction (complete depletion in some cases) of the $CD8^+$ cell population to allow survival of islet allografts. However, repopulation of these cells was observed post-moAb treatment in PBL from thymic precursors with subsequent failure of all but one islet graft and suggesting the role of these cells in the induction of an immune response against transplanted tissue in the BB/E rat. The lymphocytes and lymphocyte subsets profile of the single rat maintaining a long-term islet graft was not significantly different from rats with early graft failures or short-term functioning grafts. However, the role of $CD8^+$ cells to mediate allograft rejection remains controversial. Both $CD8^+$ and

$CD4^+$ T cells are required for the development of IDDM in NOD mice (414, 415, 875), although $CD8^+$ T cells appear to facilitate the initiation of disease rather than act as immunological effector cells. However, using a moAb depletion protocol effective in preventing transfer of diabetes (a process requiring both $CD4^+$ and $CD8^+$ T cells), Wang et al (876) examined the T cell requirement for recurrence of IDDM upon grafting syngeneic NOD mouse islets to spontaneously diabetic NOD mice. Recurrence of disease was shown to be a $CD4^+$ (but not $CD8^+$) T cell-dependent process. Depletion of the $CD4^+$ T cell subset facilitated graft survival and islets continued to function in the absence of circulating $CD4^+$ T cells. Depletion of $CD8^+$ T cells which was effective in blocking the initiation of disease in NOD mice had no effect on disease recurrence following grafting of islet tissue which became heavily infiltrated by inflammatory cells and became non-functional. FACS analysis demonstrated large numbers of $CD4^+$ T cells in the damaged tissue following $CD8^+$ T cell depletion (876). Similarly, protection against development of diabetes in the spontaneously diabetic BB/Wor rat by three-times weekly injections of tissue culture supernatant containing OX8 moAb demonstrates the role of $CD8^+$ cells in pancreatic islet β-cell destruction (239). However, CD8 cell surface antigen is also expressed on the surface of NK cells and since virtually all $CD8^+$ cells in the BB rat are considered to be NK cells (309) and that NK cells lyse islets *in vitro* (877), it is possible that islet destruction is mediated by NK and/or $CD8^+$ T cells. Indeed, Like et al (239) reported that NK cell numbers and activity were elevated in untreated DP-BB rats compared with DR-BB rats and OX8 moAb injections depleted NK cell activity. Jacobson et al (878) reported prevention of recurrent diabetes in BB rats following treatment of islet recipients with anti-asialo-GM1. Administration of this polyclonal Ab which binds the glucolipid asialo-GM1 present on NK cells effectively prevented autoimmune destruction of transplanted WF islets in diabetic BB rats. It should be noted that anti-asialo-GM1 is not a specific marker for NK cells since it is

also found to be expressed on activated peritoneal macrophages, thymocytes and cytotoxic T lymphocytes. However, markedly reduced NK cell activity in anti-asialo-GM1-treated rats and an absence of significant alterations in the relative percentages of T lymphocyte subsets suggests protection of intraportal islet grafts was achieved through the effect of anti-asialo-GM1 on NK cells. These results and the finding that $CD8^+$ cells were localised at the site of the insulitis lesion in DP-BB rats suggest that NK cells contribute to the mechanism of β-cell destruction in the initial destruction of the native pancreas and recurrent destruction of a transplanted islet graft. Both these studies (239,878) demonstrated success in preventing β-cell destruction using long-term treatment regimens (OX8 moAb and anti-asialo-GM1 respectively). It is not known whether complete islet graft survival would have been observed if islet-transplanted BB/E rats had received extended OX8 moAb treatment.

Reduction of $CD4^+$ T cell numbers in PBL or lymphoid tissues of diabetic BB/E rats by treatment with OX38 moAb was infrequent and inconsistent. Although >80% depletion of $CD4^+$ T cells has been described in peripheral blood of non-BB rats using a lower dose and shorter course of OX38 moAb treatment (678,679), Like et al (239) also failed to show depletion of $CD4^+$ T cells in the spleens of BB/Wor rats after three-times weekly injections of OX38 moAb for up to 14 weeks although the dose of moAb used was not specified and peripheral blood was not analysed. This study did not observe a significant reduction in the frequency of IDDM in OX38 moAb-treated BB/Wor rats. Indeed, in the current study all animals remained normoglycaemic throughout the moAb treatment period afterwhich 67% BB/E rats returned to a hyperglycaemic state. The remaining 33% of OX38 moAb-treated rats maintaining long-term functioning grafts received a 2° islet graft under the kidney capsule and remained normoglycaemic at death. An increased and/or extended dose of OX38 moAb may have caused significant reduction in $CD4^+$ T cells and provided

greater protection of the islet grafts against destruction. Seydel et al (679) observed significant reductions in $CD4^+$ T cell numbers and consequent permanent survival of intraportally transplanted islets in STZ-diabetic rats receiving short-term OX38 moAb treatment. This is in agreement with the earlier observation that in NOD mice recurrence of islet destruction results from $CD4^+$ T cell-dependent inflammatory tissue damage activated in and around the islet tissue.

Treatment with OX38 moAb significantly decreased the $CD8^+$ and $CD4^+/CD8^+$ cell populations particularly for rats treated with OX38 moAb alone compared with OX38 moAb-treated BB/E rats receiving an intraportal islet graft. In this group $CD8^+$ T cells were only significantly reduced in animals with short-term but not long-term functioning 1° islet grafts suggesting that depletion of $CD8^+$ cells did not result in additional protection in this group of OX38 moAb-treated rats. The finding that OX38 moAb significantly depletes $CD8^+$ lymphocytes suggests a degree of cross-reactivity with these cells although this observation has not been previously documented. However, the percentage of OX38 moAb-treated rats maintaining long-term functioning grafts (33%) was greater than observed in OX8 moAb-treated animals (11%). Again, no significant difference in lymphocyte and lymphocyte subset numbers in the peripheral blood and lymphoid tissues was observed between OX38 moAb-treated rats with short and long-term functioning grafts. This study suggests that OX38 moAb may bind to its appropriate target cells without depleting and thereby prolong graft survival by preventing antigen-induced T cell activation. However, the non-depleting anti-CD4 moAb W3/25 binds to the same epitope as defined by the OX38 moAb which suggests that if OX38 did function by binding to target cells without clearing then similar results should have been observed for both antibodies. This was not the case since 56% of W3/25 moAb-treated rats failed before the end of treatment compared with none in the OX38 moAb-treated group.

However, the different isotypes of these moAb (IgG1 and IgG2a respectively) may explain the variation in potency observed. Other studies have suggested that macrophages, which also express the $CD4^+$ cell surface marker and are therefore possible targets of OX38 moAb, may play a role in islet destruction. Oschilewski et al (344) demonstrated that administration of silica to young (i.e. prediabetic) BB rats almost completely prevented the development of IDDM. Silica is highly specific in its action against macrophages and protection is a direct consequence of macrophage depletion or modification. Additionally, a single dose of silica resulted in the indefinite survival of intraportally transplanted islet allografts in 44% STZ-diabetic rats. It should be noted that intraportally injected islets are highly accessible to macrophage surveillance and destruction in the liver (879). These findings indicate the important role of macrophages in the pathogenesis of IDDM both initial and recurrent islet destruction in the rat.

Combined OX8/OX38 moAb treatment significantly reduced both $CD4^+$ and $CD4^+/CD8^+$ lymphocytes and this was often accompanied by a significant reduction in lymphocytes and/or $CD5^+$ cells. Occasionally a significant decrease in $CD4^+$ T cells was observed although this was not a consistent finding. There was no significant difference in lymphocyte and lymphocyte subset numbers in PBL and lymphoid tissues of OX8/OX38 moAb-treated BB/E rats after separation into groups A, B and C. Using the combined treatment, 44% of diabetic BB/E rats maintained a long-term functioning islet graft and remained normoglycaemic following a 2° islet graft under the kidney capsule. Interestingly, 11% of OX8 moAb-treated and 33% of OX38 moAb-treated animals maintained long-term functioning islets which may suggest an additive effect upon combining these moAb. This result was in direct contrast to the report by Seydel et al (679) who observed that successful induction of long-term survival of an islet graft using OX38 moAb treatment was abrogated by

the coincident treatment of STZ-diabetic rats with depleting OX8 moAb. Seydel and colleagues concluded that a regulator CD8+ cell was necessary in the induction of anti-CD4-mediated survival of transplanted islets in this rat model. Factors such as differences in the rat strains used, intrinsic differences in moAb structure as well as differences in moAb preparation, dose and treatment regimens may contribute to the discrepancy in results.

Intraportal islet transplantation alone had no significant effect on lymphocyte and lymphocyte subset numbers in PBL or lymphoid tissues in any of the moAb-treated diabetic BB/E rats which confirmed the findings of Kuttler et al (880) who reported no correlation between changes in lymphocyte subsets and pancreatic β-cell destruction.

Stimulation indices were used in conjunction with FACS analysis to assess the proliferative ability of PBL isolated from moAb-treated diabetic BB/E rats to the T cell mitogen, Con A. Values of stimulation indices pre-moAb treatment demonstrate a significant response of PBL from these rats to Con A in agreement with Varey et al (212) who reported normal proliferative responses of diabetic BB/E rats to Con A and lipopolysaccharide. In contrast, Jackson et al (308) observed that Con A responsiveness was absent in the peripheral blood and splenic lymphocytes of BB rats which correlated directly to the absence of $CD4^+$ T cells, confirming the importance of these cells in mitogen stimulation. Indeed, $CD4^+$ T cell numbers were not reduced in the thymus of BB rats and thymic lymphocytes showed a significant increase in the ^{3}H-thymidine uptake when stimulated by Con A. Prud'Homme et al (881) also described a poor response in splenic lymphocytes of BB rat to T cell mitogens which was attributed to suppressor macrophages. These findings suggest that the ability of certain lymphocytes to significantly respond to Con A may be specific to the

Edinburgh colony of BB rats. In non-islet-transplanted BB/E rats, treatment with PBS or W3/25 or OX8 moAb had no significant effect on stimulation indices of PBL. In contrast, the stimulation indices of OX38 and OX8/OX38 moAb treated rats were significantly reduced during moAb treatment compared with values at the end of treatment although values 1 week post-moAb treatment were not significantly different from values pretreatment. Conversely, in islet-transplanted diabetic BB/E rats all moAb treatments caused a significant and persistent decrease in stimulation indices of PBL until death. Values were higher in group C rats (i.e. rats maintaining long-term functioning intraportal islet grafts) confirming that the immunosuppressive effect of moAb decreased with time. Only W3/25 moAb treatment which has been shown to inhibit T cell activation *in vitro* (686) significantly decreased the stimulation index of PBL prior to transplantation of 1° islet grafts compared with pre-moAb treatment. The stimulation indices confirm the FACS analysis since there was no significant difference in stimulation indices values between groups A, B and C. However, PBL of animals with a short-term functioning islet graft usually had a higher stimulation index than those with a long-term functioning graft suggesting that graft failure is accompanied by a return of the proliferative ability of PBL in BB/E rats. PBS treatment (control) also significantly reduced the stimulation indices after transplantation of an islet graft and values remained significantly lower than pretreatment values at death. This observation may involve the immunosuppressive effect of hyperglycaemia in BB rats as reported by Hahn et al (882). The decrease in stimulation index of PBS-treated BB/E rats does not reach significance compared with the value prior to PBS injections until failure of the islet graft, i.e. upon a return to a hyperglycaemic state. Hahn and colleagues reported that delayed rejection of allografted skin in hyperglycaemic BB rats compared with normoglycaemic BB rats was accompanied by an altered subset distribution and reactivity of PBL. In this study, islet graft failure in PBS-treated control rats could have an immunosuppressive

effect on PBL and hence reduce stimulation index values. Hyperglycaemia may also contribute to suppression of PBL and hence reduction of stimulation indices in short-term functioning grafts.

Histological examination of native pancreases of established diabetic BB/E rats used in this study confirmed that destruction of islets was β-cell specific since α-cells remained intact and were strongly positive for glucagon. In PBS-treated control rats, all intraportal islet grafts failed within 6 days of transplantation despite the marked T cell lymphopenia associated with a reduction of $CD4^+$ cells and almost complete depletion of $CD8^+$ cells in BB rats. Intraportal islets were chronically infiltrated by inflammatory cells, showed degenerative changes and were faintly positive or negative for insulin. Interestingly, all islet cells were negative for glucagon which suggested islets were being destroyed by allograft rejection rather than by recurrence of β-cell specific autoimmune destruction. This finding is in agreement with the hypothesis that MHC-incompatible islets are not destroyed following transplantation to the BB rat but contrast the findings of Weringer and Like (651) and Prowse et al (423) who reported that the autoimmune destruction of grafted β-cells in the BB/W rats was not confined to cultured islets of MHC-compatible donors but also occurred in grafted cells of MHC-incompatible donors. Glucagon-secreting α-cells may also have been damaged by collagenase digestion during the isolation of islets (since these cells are located at the periphery of the islet cell) or as a result of ischaemia during the 7 day culture period at 37°C prior to transplantation, both of which would contribute to the lack of glucagon-containing cells. A similar phenomenon was noted in islet-transplanted BB/E rats receiving moAb treatment irrespective of the length of survival of intraportal islet grafts, again suggesting allograft rejection rather than recurrent autoimmune destruction of islets despite the presence of the $RT1^u$ haplotype which the autoimmune response and subsequent β-cell damage

characteristic of the BB rat are reported to be dependent (338,645). However, glucagon-staining was occasionally observed but never in the absence of insulin-containing β-cells. Millard et al (883) reported similar findings following successful allografting of islets in rats with subsequent long-term survival. Islet β-cells, but not glucagon-containing α-cells, were identified suggesting this observation is not BB rat specific or a result of depleting or non-depleting moAb treatment. It is possible that α-cells (which constitute approximately 15% of the islet cells) are initially destroyed by allograft rejection due to their peripheral location in the islet or due to a greater sensitivity to immune rejection.

Intraportally-injected islets were found in the portal triads of all recipient livers upon examination and those from animals in group A were generally disaggregated and islet fragments were surrounded by a chronic inflammatory infiltrate comprising lymphocytes and macrophages. In group B, intraportal islets showed faint positivity for insulin but were rarely positive for glucagon-staining. Any glucagon positivity was generally associated with macrophages demonstrating engulfment of glucagon-containing islet fragments by these cells. This observation provides further evidence that absence of glucagon-containing α-cells may be due to early destruction by allograft rejection. In contrast, islets taken from animals in group C were only associated with a mild infiltrate in liver sections and were strongly positive for insulin but only positive for glucagon in infiltrating macrophages. Although a mild immune reaction was observed around islets in these animals, this infiltrate was not sufficient to cause destruction of islets. However, 2° islet grafts transplanted under the kidney capsule were not similarly protected and a heavy chronic inflammatory cell infiltrate was observed in and around islets which was similar in severity to that observed in islet grafts transplanted under the kidney capsule of diabetic BB/E rats receiving no moAb treatment. These results do not support the hypothesis that the kidney capsule

of diabetic BB rats is an immunologically privileged site with respect to protection of transplanted islet grafts (652).

Culture for 7 days at 37°C did not protect islets from allograft rejection as evidenced by rapid graft destruction following transplantation to PBS-treated rats. Low temperature culture (26°C) of donor rat islets has been reported to markedly prolong islet allograft survival when transplanted into STZ-diabetic rats (618) which may reflect impaired antigen presentation in the recipient due to depletion or alteration of putative passenger lymphoid cells within donor islets. However, Woehrle et al (652) reported that culture of Lewis rat islets at 22°C failed to prolong allograft survival after intraportal transplantation into diabetic BB rats. Culture in 95% oxygen and UV irradiation also immunomodulate donor islets in the same way. Both Markmann et al (682) and Jacobson et al (878) induced neonatal tolerance to donor islet tissue prior to transplantation in the BB rat to prevent the possibility of allograft rejection.

In this study the immunosuppressive potentials of both depleting and non-depleting anti-CD4 and depleting anti-CD8 moAb to promote intraportal islet graft survival were assessed. Treatment with a depleting anti-CD4 moAb (OX38) either alone or in combination with a depleting anti-CD8 moAb (OX8) was most effective at prolonging islet graft function. FACS analysis of lymphocytes and lymphocyte subsets in PBL and lymphoid tissues of islet-transplanted rats receiving moAb treatment did not reveal any consistent finding which could explain the survival of certain islet grafts but not others. However, it should be noted that analysis of lymphocytes and lymphocyte subsets in peripheral blood or lymphoid tissues may not accurately reflect events taking place within transplanted islet grafts. Similar observations were made upon analysis of stimulation indices. Histological examination of intraportal islet grafts showed evidence of allograft rejection but

recurrence of autoimmune destruction was absent. The immune infiltrate was observed within islets and was more severe in short-term functioning grafts compared with the mild infiltrate observed around islets of long-term functioning grafts.

Selective immunosuppression of the recipient of an islet allograft may therefore be a viable option when considering treatment of human IDDM. However, promising moAb therapies need to be developed in large animal models before being considered for clinical application. Strategies to prolong renal allograft survival in the dog using anti-CD4 and anti-CD8 have recently been evaluated (884). MoAb therapy had to be stopped prematurely (10 days) following adverse reactions associated with the recipient developing an antibody response against the foreign (rat) therapeutic moAb. In contrast, anti-CD4 moAb at maximal cell-depleting doses suppressed the immune response against itself in a rat model (678). Despite stopping therapy prematurely, blood levels of anti-CD4 and anti-CD8 moAb indicated that saturating doses were achieved. Neither moAb alone significantly prolonged allograft survival and renal grafts were rejected after 7 days. However, combined anti-CD4 and anti-CD8 moAb resulted in good graft function for a median of 14 days even though the reduction in $CD4^+$ cells in the peripheral blood was only approximately 50%. Since only a fraction of the normal T cell population is required to initiate immune rejection of an allograft (676), the action of the anti-CD4 moAb was thought to involve coating of the target T cell population. In contrast, anti-CD8 moAb caused >90% depletion of $CD8^+$ T cells from the peripheral blood. Although allograft survival was prolonged in the above study, a more extensive graft prolongation is necessary for the development of specific immunosuppression therapies. A similar protocol using a mouse anti-human CD4 moAb, OKT4A in cynomolgus monkeys prolonged allograft survival to a median of 23 days compared with rejection by day 11 in untreated control monkeys (885). A single high dose (10 mg/kg) of OKT4A on

the day of transplantation also resulted in prolonged renal allograft survival in monkeys further illustrating the therapeutic efficacy of CD4. The beneficial effect observed when anti-CD4 moAb was combined with conventional immunosuppression in the primate has also been described (886). A mixture of depleting and non-depleting anti-CD4 moAb (OKT4 and OKT4A) was tested for its immunosuppressive potential in rhesus monkeys receiving a kidney allograft in combination with low dose azathioprine and prednisolone to mimic the clinical situation. Administration for 21 days, starting 2 days before transplantation of the kidney graft prolonged graft survival from 13 days in animals receiving azathioprine and prednisolone alone to 39 days. These studies demonstrate that at this time, immunosuppression of an allograft in large animal models using moAb is difficult to achieve. Although long-term acceptance of grafted tissues in humans in the absence of current and continued immunosuppression and its related side effects is the goal of moAb therapy, additional immunosuppressive drug therapy may still be required until more efficient moAb have been developed.

CHAPTER 5

FINAL DISCUSSION

Three possible new approaches to the treatment of human IDDM using the spontaneously diabetic, insulin-dependent BB/E rat as a model for the human disorder have been studied. Although the BB rat is an invaluable model for human IDDM, ultimately promising therapies need to be developed in larger animal models prior to consideration for clinical application.

SRII have been shown to successfully normalise the diurnal variation in plasma glucose concentrations observed in CIT-treated diabetic BB/E and STZ-diabetic rats and parameters of metabolic control (i.e. random plasma glucose concentration and HbA_1) are accurately reflected in such animals. The impaired control observed with CIT treatment may contribute to the long-term complications and morbidity associated with IDDM. Despite the improved glycaemic regulation achieved by SRII the resulting reduction in tissue concentration of the principal metabolites of the polyol pathway was similar in both SRII- and CIT-treated STZ-diabetic rats. The prospects for the clinical treatment of IDDM using SRII are encouraging, although a dual implant scheme may be necessary. The observed success of SRII in the maintenance of glucose homeostasis in the BB/E rat model was related to the finding that these animals ate throughout the 24 hour period and therefore a constant basal release of insulin was sufficient to maintain normoglycaemia. In human diabetic subjects, insulin demands vary with meal intake and physical activity and supplemental doses of insulin in addition to the basal dose of insulin provided by long-acting SRII will be necessary. For the open-loop arrangement, this pre-prandial insulin bolus may be supplied by an implantable osmotic pump or injections. Similarly, implants with internal modulation capability can be used as the required component in a closed-loop system to provide the additional doses of insulin required to counter post-prandial hyperglycaemia.

Encapsulation of pancreatic islets in semipemeable APA membranes has been used as a method of protecting islets from immune destruction in transplantation studies. Insulin secretion by microencapsulated islets was optimal after 7 days culture and diminished thereafter, suggesting a precisely timed recovery culture period prior to implantation is important to ensure optimal function of encapsulated islet grafts. However, upon implantation into spontaneously diabetic animals, early capsule failure was observed. Failure was ascribed to fibrotic overgrowth around the capsule surface activated by the incompatibility of reagents used to construct the membrane. This pericapsular infiltrate resulted in necrosis of islets due to lack of oxygen and nutrients and possible release of β-cell toxic cytokines from components of the immune response which are not excluded by the capsule membrane. Immunosuppressive agents such as CsA reduce the amount of capsular infiltrate and clinically, the only successful report describing insulin-independence after microencapsulated islet transplantation has been in an immunosuppressed IDDM patient. Although encouraging, further trials to determine the optimal dose of encapsulated islets necessary to achieve long-term insulin-independence in non-immunosuppressed patients are required.

Rejection and/or autoimmune destruction of transplanted islets may also be prevented using depleting and non-depleting moAb specific for the T cells responsible for immune destruction. Successful protection and prolongation of survival of intraportal islet grafts in the BB/E rat was observed with a depleting anti-CD4 moAb (OX38) either alone or in combination with a depleting anti-CD8 moAb (OX8). All islet grafts showed a degree of inflammatory cell infiltrate, the intensity of which was reflected by the period and degree of normoglycaemia observed in the recipient. Short-term functioning islet grafts were heavily infiltrated and showed weak or no positivity for insulin whereas only a mild infiltrate was observed around islets of

long-term functioning grafts and these were strongly positive for insulin. Secondary islet grafts transplanted under the kidney capsule of BB/E rats maintaining long-term functioning intraportal grafts were not afforded similar protection and islets were heavily infiltrated, demonstrating treatment had not initiated long-term unresponsiveness to donor-specific islets. Although similar reports of prolonged tissue allograft survival using moAb therapy are well documented in rodents, studies with larger animal models including dog and monkey have had limited success. Adverse reactions associated with an antibody response against the foreign therapeutic moAb are observed in the recipient demonstrating the need to render such moAb invisible to the immune systems of large animal models and humans. Antibody engineering to humanise therapeutic antibody has reduced the risk of immunogenicity despite the lack of preclinical information available on therapeutic moAb. However, attempts to simulate the rodent data using anti-CD4 and anti-CD8 moAb therapy have not been successful. The anti-CD3 moAb, OKT3 has been most widely used and is effective in reversal of acute rejection episodes both as an initial treatment and for patients unresponsive to high-dose steroid therapy. There has also been interest in targeting activated T cells as these cells must include the antigen-reactive cohort. IL-2R is an obvious choice of target, but limited clinical studies using prophylactic IL-2R moAb have given mixed results. Initial observations in large animal models and humans suggest that although it is too early to make a compelling case for moAb therapy for the prevention of of allograft destruction, it is possible that moAb could be combined synergistically with additional currently used immunosuppressive drug therapies.

5.1. OVERALL CONCLUSION

Several factors have recently underlined the urgent need for a better treatment for IDDM. These include (1) the high incidence of severe vascular disease seen in patients maintained on conventional insulin replacement therapy, (2) an increased incidence of IDDM in Northern Europe and America, (3) the continuing high foetal loss rate in women with IDDM, (4) the conclusive evidence provided by the DCCT not only that the long-term complications of diabetes results from the far from normal metabolic regulation seen in most patients maintained on conventional insulin replacement therapy, but also that the complications can be prevented by good metabolic control, and (5) that the inherent limitations of conventional insulin therapy means that achievement of good control is often accompanied by an increased incidence of serious hypoglycaemic episodes and imposes unrealistic demands on patients by the need for multiple daily injections of insulin, dietary measures and self-measurement of capillary blood glucose concentration.

The availability of valid animal models for human IDDM along with recent advances in key areas such as genetic engineering, induction of peripheral tolerance to autoantigens, understanding of the mechanisms underlying β-cell destruction and of the factors regulating growth and differentiation of islet cells make it possible to pursue new approaches to more effective management of patients with IDDM.

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